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Growth and Reproduction During Chronic Exposure to Carbon Monoxide.*

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In the course of studies on the role of heredity in determining individual differences in resistance to anoxia, observations have been made on growth and reproduction of rats during chronic anoxia produced by exposure to carbon monoxide. These experiments differ importantly from similar ones reported by other workers[1,2,3,4] in that the concentration of carbon monoxide (0.04%) was below the level at which acute toxic symptoms or general impairment of health occur.

Monge[5] discusses at length the deleterious influence on fertility resulting from residence at high altitudes. Miscarriages, abortions, sudden death of the new-born and complete sterility have been reported, both for the human and for domestic animals. In many cases (perhaps all) normal fertility is restored on return to lower altitudes. A fact of great interest is that the native inhabitants of high altitudes (both man and domestic animals) have normal fertility, presumably the result of a slow process of acclimatization requiring generations for completion.

The authors know of no reports of impairment of fertility resulting from stagnant or histotoxic anoxia.

Methods. Newly weaned rats (Vanderbilt

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1 Buresch, H., *Arch. f. Hyg.*, 1933, **109**, 211.

2 Campbell, J. A., *Quart. J. Exp. Physiol.*, 1934, **24**, 271.

3 Williams, I. R., and Smith, E., *Am. J. Physiol.*, 1935, **110**, 611.

4 Killick, E. M., *J. Physiol.*, 1937, **91**, 279.

5 Monge, C., *Physiol. Rev.*, 1943, **23**, 166.

strain) were used. Litter mates were divided into 2 equal groups for experimental and control animals. A total of 24 males and 24 females was used for each group. The experimental animals were exposed to carbon monoxide, by a method previously described,[6] 8 hours daily, except Saturday and Sunday.

Results. Sixteen of the control females have borne litters; 4 of them have borne 2 litters each. Of the 20 litters, 16 were weaned, averaging 5.0 to a litter (all litters were reduced to 6 at birth). This is somewhat below normal fertility, but the rats were taken from a newly established colony, unselected for breeding. During this same period there were 15 pregnancies in the experimental group. Four of these terminated in abortion and 11 litters were delivered at term. It was impossible to determine accurately the number of young in each litter, because the mother usually ate some of the young soon after birth. The average litter size (including stillborns) was 3.9. The average weight of the young was 3 to 5 g, while the average weight for the young of control animals was 5 to 7 g. In every case the mother refused to nurse her young, and death of all the young usually occurred in 1 to 3 days; the longest survival period was 8 days.

The growth curves for experimental and control animals were identical within limits of normal variation. At 114 days the average weights were: male controls 310 g, male experimentals 300 g, female controls 176 g, female experimentals 179 g. These results differ from those of Campbell,[2] in which much higher concentrations of carbon monoxide were used. He observed that during the first 100 days, when acclimatization was developing, the mortality rate was high and growth lagged behind that of the controls. Killick[4] observed a loss of weight in adult mice during acclimatization to gradually increasing concentrations of carbon monoxide.

Discussion. Our results emphasize the importance of Campbell's[2] insistence that acclimatization to carbon monoxide (or to any form of anoxia) cannot be considered complete unless fertility (pregnancy leading to the birth of normal young) is unimpaired. We would like to suggest, tentatively, that normal lactation leading to the weaning of healthy young be included, since, in at least one of our cases, a litter of normal size and average weight failed to nurse after 5 days and died in 8 days. Further studies are needed to determine the cause of the invariable failure of mothers exposed to carbon monoxide to nurse their young.

Conclusions. Rats exposed daily to carbon monoxide (0.04%) grow normally and have about the same incidence of pregnancy as do control litter mates. However, there is a tendency toward abortion, the average size of the litter and the average weight of the young at birth are subnormal, and the mothers invariably fail to nurse their young. The cause of this lactation deficiency is under investigation.

[6] Miller, A. T., Jr., *J. Lab. and Clin. Med.*, 1943, **28**, 1854.

14470

Acetylation in Patients with Myasthenia Gravis. The Elimination of Para-amino Benzoic Acid.

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The weakness of certain skeletal muscles and the easy fatiguability observed in patients with myasthenia gravis is probably due to an insufficient production of acetylcholine (Torda and Wolff[1]). This decreased synthesis of

[1] Torda, C., and Wolff, H. G., *Science*, 1943, **98**, 224.

TABLE I.
Short Summary of the Clinical State of the Patients with Myasthenia Gravis.

Name	Sex	Age	Severity of disease	Duration (years)	Thymectomy	X-ray treatment	Symptomatology	Neostigmine (Prostigmine bromide) (Hoffmann-LaRoche)		
								Dose before thymectomy mg/day	Dose mg/day	Achievement after medication
H	F	43	5+	15	yes	yes	Bedridden, severe lid ptosis, diplopia, constant difficulty in chewing and swallowing, very severe muscular weakness	225	120-180	Chews food, turns in bed, sits on chair
R	F	23	3+	9	no	"	Moderate lid ptosis, occasional diplopia, occasional difficulty in chewing, moderate muscular weakness		90	Walks 1-2 blocks
Sa	F	32	3+	7	"	"	Moderate lid ptosis, occasional diplopia, occasional difficulty in chewing, moderate muscular weakness		90-150	Housework
S	M	22	2+	2	yes	no	Slight lid ptosis, rare diplopia, moderate muscular weakness	120	15-45	Works as an inspector
N	F	47	1+	12	no	"	Mild muscular weakness		30	Housework

acetylcholine may be due to one or more factors. Insufficient amounts of basic precursor substances, namely, choline and the acetyl- radical, is one of these possible factors. The purpose of the following investigation is to ascertain whether processes of acetylation are defective in patients with myasthenia gravis. Since acetylation of choline and acetylation of other chemically different substances have probably in common only the use of the acetyl- radical, faulty acetylation of other substances than choline would indicate that insufficient amount of acetyl- radical was available. Therefore, if an agent were administered that was eliminated in acetylated form in healthy subjects, the success or failure of patients with myasthenia gravis to achieve acetylation would be an indication of the ability of the patients to complete such a process. From the group of substances which may be acetylated by man if taken by mouth, *p*-amino benzoic acid[2-6] was selected for this investigation.

Method. *P*-amino benzoic acid was administered orally in both small and large doses to both healthy subjects and to patients with myasthenia gravis, and the elimination of free and acetylated *p*-amino benzoic acid was determined. Summaries of the clinical state of the patients with myasthenia gravis are presented in Table I.

I. *Small Doses.* 300 mg of *p*-amino benzoic acid were orally administered in a single dose to healthy subjects and to patients with myasthenia gravis. Separate specimens of urine were collected over a period of 24 hours. The free and conjugated *p*-amino benzoic acid content of the separate samples were ascertained following the method of Bratton and Marshall.[7]

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3 Hensel, M., *Z. f. physiol. Chem.*, 1915, **93**, 401.

4 Cerecedo, L. R., and Sherwin, C. P., *J. Biol. Chem.*, 1924, **62**, 217.

5 Muenzen, J. B., Cerecedo, L. R., and Sherwin, C. P., *J. Biol. Chem.*, 1924, **67**, 217.

6 Ambrose, A. M., and Sherwin, C. P., *Ann. Rev. of Biochem., Stanford University*, 1933, **2**, 393.

7 Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, **128**, 537.

TABLE II.
Excretion of 300 mg *p*-Amino Benzoic Acid.

Hr after ingestion	Control subjects								Patients with myasthenia gravis					
	G	T_1	W	T_2	Su	Be	Si	La	We	Wo	Me	T_3	H_1	H_2	R	S	N
									Free *p*-amino benzoic acid								
2	25.0	7.7	19.0	13.3					14.9		15.7	12.0		16.0			
3																15.0	
4	1.2	6.3	3.8	11.3	18.0		20.0		12.6	41.0		3.7					11.4
6	1.0	7.0	0.2	3.4				25.0			18.4			14.3	26.0		
8	0.5	2.5	1.0			17.2	12.0		0.9			1.5	36.2				4.6
10	0.5	0.2	0.4	0.8			2.4	2.0					2.0	2.2		6.8	
12		0.6	0.2									2.8	1.2	0.4			0.7
16	0.8						4.2	3.0									0.9
22		1.1	0.3	0.9	6.0	6.7		4.5						1.2		1.9	2.0
Total	29.0	25.4	24.9	29.7	24.0	23.9	38.6	34.5	(28.4)	(41.0)	(34.1)	20.0	39.4	34.1	(26.0)	23.7	19.6
									p-Acetylamino benzoic acid								
2	42.0	41.9	36.2	19.5					62.0		34.0	31.2		39.0			
3																46.0	
4	86.0	83.0	52.6	35.4	104.0		105.0		57.0	130.0		75.6					69.0
6	44.5	36.2	37.0					161.0			80.0			77.0	154.0		
8	11.6	6.4	34.0	94.2		148.8	49.8	43.6	33.7			52.5	119.5				72.0
10	3.9	8.0	14.2	20.7			19.2						34.0	50.0		133.0	
12		2.0	5.0	8.0								40.5	34.0	6.6			35.0
16	6.7						15.5	4.0									6.8
22		3.5	3.5	3.7	96.2	30.4		3.0				10.0		0.3		12.0	2.8
Total	194.7	181.0	182.5	181.5	200.2	179.2	189.5	211.6				209.8	187.5	172.9		191.0	185.6

TABLE III.
Excretion of 2 g *p*-Amino Benzoic Acid.

Subject	Excreted in mg	
	p-amino benzoic acid	*p*-acetylamino benzoic acid
Healthy:		
T_1	1000	680
T_2	1120	580
Myasthenia gravis:		
Sa	876	684
R	1085	650

II. *Large Doses.* 2 g of *p*-amino benzoic acid were orally administered in a single dose to both healthy subjects and patients with myasthenia gravis. Urine was collected over a period of 48 hours. An aliquot was taken to ascertain the amount of free and conjugated *p*-amino benzoic acid excreted (method of Bratton and Marshall[7]). The conjugated *p*-amino benzoic acid was isolated from the remainder following the method described by Harrow and Mazur[8] and the identity of the isolated compound with *p*-acetylamino benzoic acid was ascertained by determination of the melting point.

Results. I. *Excretion of Small Doses of* p-*Amino Benzoic Acid.* Seventy per cent of the 300 mg of *p*-amino benzoic acid taken in a single dose was excreted during the first 24 hours. On the average 45% was excreted in conjugated form and the remaining in free form as shown in Table II. Both healthy subjects and patients with myasthenia gravis excreted *p*-amino benzoic acid in essentially the same manner in respect to time of elimination and amount of free and conjugated compound.

II. *Excretion of Large Doses of* p-*Amino Benzoic Acid.* Eighty per cent of the 2 g of *p*-amino benzoic acid were excreted during the first 48 hours. On the average 34% was excreted in conjugated form and the remainder in free form as shown in Table III. Patients with myasthenia gravis excreted this large dose of *p*-amino benzoic acid in a manner similar to healthy persons. However, it seemed to be necessary to ascertain whether the conjugated compound was *p*-acetylamino benzoic acid in both cases. Therefore the compound was isolated and the melting point determined. Since the melting points of the isolated compounds were 248°C in both cases, it is likely that *p*-amino benzoic acid was eliminated as *p*-acetylamino benzoic acid in both healthy subjects and patients with myasthenia gravis.

Discussion. Both patients with myasthenia gravis and healthy subjects excreted *p*-amino benzoic acid in essentially the same manner in respect to time of excretion and amounts of free and conjugated (acetylated) form. A faulty acetylation resulting from insufficient acetyl- radical would be more easily detected with large doses of *p*-amino benzoic acid taken because of the increased requirement. Since both healthy subjects and patients with myasthenia gravis excreted similar amounts of *p*-amino benzoic acid, it may be assumed that there is no significant insufficiency of the acetyl- radical in patients with myasthenia gravis.

The acetylation of *p*-amino benzoic acid is not necessarily a measure of the acetylation of choline, and probably involves entirely different processes. The acetylation of *p*-amino benzoic acid occurs mainly in the liver[6] while the acetylation of choline occurs mainly in the nervous tissue. The enzymes involved and the oxygen requirements of the two processes may also differ. However, an undisturbed and ready acetylation of *p*-amino benzoic acid even in large amounts suggests that no severe insufficiency of the acetyl-radical occurs in patients with myasthenia gravis. A similar conclusion may be drawn from the clinical observation that patients with myasthenia gravis do not benefit significantly from the administration of insulin and glucose.

Summary. 1. The excretion of both small and large amounts of *p*-amino benzoic acid

[8] Harrow, B., Mazur, A., and Sherwin, C. P., *J. Biol. Chem.*, 1933, **102**, 35.

administered orally in a single dose to healthy subjects and to patients with myasthenia gravis was investigated. 2. Both healthy subjects and patients with myasthenia gravis excreted *p*-amino benzoic acid in essentially the same manner in respect to time of elimination and amounts of free and acetylated form. 3. It is inferred that there is no serious insufficiency of acetyl- radical in patients with myasthenia gravis.

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Action of Sulfonamides on Toxins of Agents of the Lymphogranuloma-Psittacosis Group.

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It has been shown elsewhere[1,2,3] that all four of the agents of the lymphogranuloma-psittacosis group[4] investigated so far produce a rapidly lethal toxin which in general resembles the bacterial endotoxins. These toxins are distinguishable one from the other by certain characteristics of the distribution curve of time of death and by differences, in certain instances, in the pathological picture produced, as well as by the sharp specificity of the toxin-antitoxin reaction. While intravenous inoculation of the agent of lymphogranuloma venereum results only in early deaths[1,2] that could hardly be due to anything other than toxin (since 83% of deaths occur within 24 hours and 95% within 36), the other 3 toxic materials produce diphasic distribution curves of time of death, the early curve of toxicity being followed by a later curve in which deaths are due to infection.[2,3]

Following the earliest report on the existence of these toxins the question has been raised as to how one could be sure that one was dealing with a toxin and not with an infection which was acting very rapidly. This point, while hardly valid in connection with the very early deaths, did seem to be pertinent in those cases where toxemia was followed, often without any distinct interval (*i.e.*, in the case of meningopneumonitis), by deaths certainly due to infection. Could one distinguish clearly between the two forms of death?

It has been possible to demonstrate that infection with 2 of these agents, namely, lymphogranuloma venereum and mouse pneumonitis, is controllable with sulfonamides[5,6] while the infection with feline pneumonitis (Baker[7,8]) and meningopneumonitis is not.[3,6] If the theories, which have been postulated elsewhere[1,2,3] and set out briefly above, concerning the cause of deaths following intravenous inoculation of mice with these toxins are correct it should be possible to demonstrate this fact by means of sulfonamide therapy. In the case of lymphogranuloma venereum only supposedly toxic deaths occurred and these should be unaffected by sulfonamides. In the case of mouse pneumonitis where there is a distinct interval between the early, supposedly toxic, deaths and the later infectious deaths the later deaths should be eliminated by sulfonamide therapy while the early deaths should not. Finally, meningopneumonitis and feline pneumonitis both give diphasic curves of death and the infectious deaths follow closely upon the toxic. Infections due to these agents are not affected

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TABLE I.
Effect of Sulfamerazine on Toxic and Infectious Deaths Following Intravenous Inoculation of L.V., M.P., and F.P.

Agent	Dilution	Control Sherman diet	Sulfamerazine 0.1% in diet
L.V. (Watson)	1/10	<17,* <17, <17, <17, <17, <17, <17, <17, <17, <17	<17, <17, <17, <17, <17, <17, <17
	1/40	<17, <41, <41, <41, S,* S, S, S, S, S	<17, <17, 25, <41, S, S, S, S, S, S
M.P. (Atherton)	1/5	<16, <16, <16, <16, **40, 43, <64, <88, <6d,** S	<16, <16, <16, S, S, S, S, S, S, S
,, ,,	1/5	1, 1½, <18, <18, <18, <18, <18, <18, <18, <18	1, 1½, 1½, 2, <18, <18, <18, <18, <18, <18
	1/7.5	23, **<46,* <46, <46, <46, <46, 47, 49, 50, 51, 51, 54, 56, 56, 56, <70, <70, <70, <70, <94**	S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S
F.P. (Baker)	1/40	<18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18	<18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, <18, 22, 22
,, ,,	1/320	**48, <62,** S, S, S, S, S, S, S, S, S, S, S, S, S, S, S	**54, <62, 70, <86, <6d,** S, S, S, S, S, S, S, S, S, S, S, S, S, S, S, S
,, ,,	1/240	**<65, <65, <89, <5d,** S, S, S, S, S, S, S, S, S, S, S, S, S, S, S	**<66, <66, <66, 73, 74, 76,** S, S, S, S, S, S, S, S, S, S, S, S

<17 = died in less than 17 hours.
S = survived for over 10 days.
<46 = died in less than 46 hours. Figures given in heavy type indicate deaths which in previous work and on the basis of other data[1,2,3] have been considered due to infection and not to toxemia.

by sulfonamide therapy and, therefore, one would expect the curves of death to be unaffected by such therapy.

Experiments were set up with 3 agents showing the 3 different types of response, namely, lymphogranuloma venereum (L.V.), mouse pneumonitis (M.P.), and feline pneumonitis (F.P.). The results are shown in Table I and completely confirm the theories postulated above. Thus sulfamerazine has no effect upon the early toxic deaths in the case of any agent even when only a border line dose is used. The late infectious deaths are eliminated in the case of mouse pneumonitis and are not affected in the case of feline pneumonitis even when border line doses are used.

Summary. Sulfamerazine has no effect on any of the endotoxins of the agents of the lymphogranuloma-psittacosis group and on the early deaths produced by such toxins, even when these are used in doses on the border line of lethality and are derived from lymphogranuloma venereum or mouse pneumonitis both of which infections are very susceptible to sulfonamide therapy. On the other hand, of the later deaths which occur following intravenous inoculation of the agents of mouse or feline pneumonitis, those occurring with mouse pneumonitis are entirely prevented with sulfamerazine while those with feline pneumonitis are not. These facts are in keeping with the previously postulated theory that these later deaths are due to infection while the earlier are due to toxemia.

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Influence of Certain Purines, Pyrimidines, and Pterins on Synthesis of "Folic Acid" by *Aerobacter aerogenes.*

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Snell and Peterson[1,2] reported that *L. casei* requires for growth a hitherto unrecognized nutrilite which they termed the *Lactobacillus casei* eluate factor. They noted that the material possessed many of the properties expected of a naturally occurring purine. Subsequent papers from other laboratories have corroborated the purine-like nature of the material. Thus Stokstad[3] found that a combination of guanine and thymine possessed some growth-promoting property in lieu of concentrates of the eluate factor. Mitchell, Snell, and Williams[4] found that *Strep. lactis R* requires an essential growth factor, concentrates of which would likewise promote growth of *L. casei.* The factor was concentrated to a state approaching purity and was named "folic acid." Thymine and uric acid each had some slight "folic acid" activity.[5] Recent evidence indicates that there are several naturally occurring materials which vary in their effectiveness as growth factors for *L. casei* and *Strep. lactis.*[6,7]

In experiments with surviving rat liver it has been shown that following the incubation of rat liver with xanthopterin more "folic acid" is found on microbiological assay of the digestion mixture than is present in a similar amount of rat liver alone.[8,9] This finding appears to indicate that under these conditions xanthopterin in some manner influences the synthesis, destruction, or other reactions of "folic acid." Conceivably xanthopterin or some closely related derivative either constitutes one moiety of the "folic acid" molecule, or, by virtue of structural similarity, interferes with "folic acid" metabolism. Totter and Day[10,11] have also provided evidence for a relationship between "folic acid" and xanthopterin.

In an attempt to obtain further information on the structure of "folic acid," the influence of various purines, pyrimidines, and pterins on the synthesis of "folic acid" by *Aerobacter aerogenes* has been investigated. This organism was found[12] to produce large amounts of "folic acid," when grown aerobically in a medium of glucose, hydrolyzed vitamin-free casein, and inorganic salts.

The media used were prepared as follows:

	A	B	C
Glucose	4 g	4 g	4 g
Salts A [13]	1 ml	1 ml	1 ml
Salts B [13]	1 "	1 "	1 "
Salts 1 [14]	0.2 "	0.2 "	0.2 "
Salts 2 [14]	0.2 "	0.2 "	0.2 "
Hydrolyzed casein (SMA Corp.)	0.2 "	0.8 "	2.0 "
Water to	200 "	200 "	200 "

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2 Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 273.

3 Stokstad, E. L. R., *J. Biol. Chem.*, 1941, **139**, 475.

4 Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. A. C. S.*, 1941, **63**, 2284.

5 Mitchell, H. K., and Snell, E. E., *The University of Texas Publication*, 1941, **4137**, 36.

6 Keresztesy, J. C., Rickes, E. L., and Stokes, J. L., *Science*, 1943, **97**, 465.

7 Stokstad, E. L. R., *J. Biol. Chem.*, 1943, **149**, 573.

8 Wright, L. D., and Welch, A. D., *Am. J. Med. Sci.*, 1943, **206**, 128.

9 Wright, L. D., and Welch, A. D., *Science*, 1943, **98**, 179.

10 Totter, J. R., Shukers, C. F., Kolson, J., Mims, V., and Day, P. L., *Fed. Pro.*, 1943, **2**, 72.

11 Totter, J. R., and Day, P. L., *J. Biol. Chem.*, 1943, **147**, 257.

12 Thompson, R. C., *The University of Texas Publication*, 1942, **4237**, 87.

13 Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

14 Williams, R. J., and Saunders, D. H., *Biochem. J.*, 1934, **28**, 1887.

TABLE I.

Influence of Certain Purines, Pyrimidines, and Pterins on Synthesis of "Folic Acid" by *Aerobacter aerogenes*. *Strept. lactis R* was used as the assay organism.

Exp. No.	Medium	Test material	Turbidity	"Folic acid," mγ/ml	Biotin, μγ/ml
1	A	None	112	3.5	—
		5γ xanthopterin	113	2.5	—
		10γ "	119	2.2	—
		20γ "	112	1.5	—
		40γ "	117	1.5	—
2	A	None	128	6.3	—
		37.5γ xanthopterin	129	3.7	—
		75.0γ "	129	3.7	—
		187.5γ "	129	3.7	—
3	A	None	116	4.0	54
		37.5γ xanthopterin	119	2.0	48
		75.0γ "	117	1.8	53
		187.5γ "	113	2.0	45
		375.0γ "	119	2.2	58
4	A	None	121	2.5	19
		100γ xanthopterin	121	1.0	19
		100γ adenine, cytosine, guanine, uracil, or xanthine	120-125	2.5	15-18
5	A	None	132	4.0	—
		100γ xanthopterin	135	1.8	—
		100γ leucopterin, iso-xanthopterin, or thymine	131-134	4.2	—
6	A	None	118	5.3	—
		100γ xanthopterin	120	2.0	—
		100γ thymine, uric acid, hypoxanthine, or allantoin	116-124	5.0-6.0	—
7	A	None	150	4.0	—
		100γ xanthopterin	151	2.5	—
	B	None	214	8.8	—
		100γ "	212	2.7	—
	C	None	248	12.7	—
		100γ "	246	4.7	—
8	C	None	212	8.8	22
		100γ xanthopterin	240	3.0	20
		100γ leucopterin, iso-xanthopterin, thymine, adenine, cytosine, guanine, uracil, xanthine, uric acid, hypoxanthine, or allantoin	216-248	9.2-11.7	18-22

The pH was adjusted to neutrality before use. Medium C is that used by Thompson.[12] Medium C permits luxuriant growth of *A. aerogenes*, medium B permits good growth, while only moderate growth results with medium A because of the small amount of nitrogen it contains. The cultures for microbiological assay were grown in 125 ml Erlenmeyer flasks. The materials to be tested were added in 10 ml of water. Of the medium (double strength), 10 ml were used and the flasks sterilized by autoclaving for 15 minutes at 15 lb pressure. One ml of a 24-hour culture of *A. aerogenes* in medium A was used for inoculation of the flasks. The cultures were incubated at 37°C for 8-24 hours, depending on the rate of growth of the organisms. After incubation the cultures were autoclaved, the bacterial density determined turbidimetrically, and the "folic acid," and occasionally the biotin, content of the combined cells and medium determined microbiologically. Enzymatic digestion of the cultures to liberate any bound "folic acid" or biotin was not employed since

Thompson has shown[12] that when *A. aerogenes* is grown aerobically most of the "folic acid" and biotin produced appears in an uncombined form in the medium and is not retained within the cells.

Bacterial density was determined in the Klett-Summerson photoelectric colorimeter. A 540 μ filter was used. The instrument was adjusted to read 0 against distilled water. Uninoculated medium read about 40. "Folic acid" was determined either with *Streptococcus lactis R* as the test organism according to the method described by Mitchell and Snell[5] or with *Lactobacillus casei* ϵ as the test organism, using the method of Landy and Dicken.[15] A concentrate of "folic acid" (about 200 Snell-Peterson units of "folic acid" per milligram) kindly furnished by Dr. E. L. R. Stokstad served as the "folic acid" standard. The standard has now been assayed, with the two organisms, against a sample of a crystalline folic acid from liver, kindly furnished by Dr. E. L. R. Stokstad, and the results have been expressed in terms of millimicrograms (10^{-9} g) of liver "folic acid" per ml of combined cells and medium.

Biotin was determined by the method of Landy and Dicken[15] using *L. casei,* or by the method of Snell and Wright[13] using *L. arabinosus.*

The xanthopterin, leucopterin, and isoxanthopterin used were kindly prepared by Drs. A. H. Land and J. M. Sprague of these laboratories, according to published methods.[16,17,18] Dr. F. S. Daft of the National Institute of Health kindly supplied the sample of thymine used. The other materials tested were commercial products.

Table I presents the results obtained.

In its effect xanthopterin was unique among the compounds tested. Although the addition of xanthopterin to cultures of *aerogenes* was without effect on the growth of the organism, or on the amount of biotin produced, the amount of "folic acid" found was invariably decreased significantly. Although the results were qualitatively similar whether performed with *S. lactis* or *L. casei,* higher values for "folic acid" content were obtained with the latter test organism. This probably indicates that the "folic acid" produced by *A. aerogenes* is more active for *L. casei* than for *S. lactis.* (Stokstad[7] found that a folic acid isolated from yeast was about twice as active for *L. casei* as for *S. lactis.*) Such an effect with xanthopterin was also observed both in the presence of rapid luxuriant growth (medium C) and in the presence of slow moderate growth (medium A). Other experiments, not reported in detail here, have shown that the xanthopterin influenced the amount of "folic acid" formed by *A. aerogenes* and that it did not inhibit the growth or acid production of the assay organisms. Derivatives as closely related to xanthopterin as isoxanthopterin (2-amino-6,9-dioxypteridin) or leucopterin (2-amino-6,8,9-trioxypteridin) were without effect on the "folic acid" production of *A. aerogenes.* None of the other purines or pyrimidines tested (Table I) had any effect on the growth of *A. aerogenes* or on its "folic acid" or biotin production.

By independent methods it has been concluded that xanthopterin, or a material closely related to it is similar in structure to one portion of the "folic acid" molecule. An explanation for the phenomenon observed might be found in the following alternatives: (1) under some conditions xanthopterin is able partially to replace "folic acid" in metabolism, (2) xanthopterin is similar enough in structure to an intermediate in the microbiological synthesis of "folic acid" that it effectively inhibits the utilization of the intermediate yet in itself is incapable of ready conversion to "folic acid." The first hypothesis is supported by the fact that xanthopterin shows no toxicity for *A. aerogenes,* as evidenced by the growth of the organism or by the biotin which it produces. The second possibility is in general agreement with much of the available information concerning vitamin antagonism where a derivative closely related to an active metabolite may prevent the normal action of the metabolite yet in itself be incapable of replacing the vitamin. Under such conditions, however, and contrary to the findings pre-

[15] Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.,* 1942, **27**, 1086.

[16] Purrmann, R., *Ann.,* 1940, **546**, 98.

[17] Purrmann, R., *Ann.,* 1940, **544**, 182.

[18] Purrmann, R., *Ann.,* 1941, **548**, 284.

TABLE II.
Comparison of "Folic Acid" Production of *Aerobacter aerogenes* as Influenced by the Presence of Various Pterins when Assays Were Performed with *Strept. lactis R* and *L. casei* ε

Exp.	Medium	Test material	Turbidity	"Folic acid" produced: *Strept. lactis* assay mγ/ml	"Folic acid" produced: *L. casei* assay mγ/ml	Biotin produced μγ/ml
1	C	None	321	9.2	16.8	15
		100γ xanthopterin	325	2.5	5.4	15
		100γ leucopterin	320	9.0	17.0	17
		100γ isoxanthopterin	321	9.2	17.6	15

sented, large amounts of the antimetabolite might be expected deleteriously to influence the growth of the organism or nearly completely to inhibit the formation of the metabolite. Obviously, an adequate explanation for the depressant effect of xanthopterin on the production of "folic acid" by *A. aerogenes* cannot be offered at this time. It is suggested, however, that these experiments supply additional evidence for relating the structure of xanthopterin to that of "folic acid." Available data on the empirical analysis of crystalline folic acids[7,19] appear to substantiate this suggestion.

Summary. When xanthopterin was added to cultures of *A. aerogenes* only small amounts of "folic acid" were synthesized by the organism. Xanthopterin was without effect on the resulting turbidity of the cultures or on the amount of biotin produced. Several other purines, pyrimidines, and pterins were without effect on the growth of the organism or on the production of "folic acid" or of biotin. It is suggested that xanthopterin either may partially replace "folic acid" in metabolism or its structural similarity to an hypothetical intermediate in the microbiological synthesis of "folic acid" enables it to inhibit the synthesis or utilization of the intermediate.

[19] Pfiffner, J. J., Binkley, S. B., Bloom, E. S., Brown, R. A., Bird, O. D., Emmett, A. D., Hogan, A. G., and O'Dell, B. L., *Science*, 1943, **97**, 404.

14473

Muscle Glycogen as Influenced by Castration, Adrenalectomy, and Treatment with Testosterone and Desoxycorticosterone Acetate.

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Schumann[1] has reported that castration of male rats and rabbits leads to a depletion of muscle glycogen. Testosterone propionate or desoxycorticosterone acetate were said to be effective in restoring the muscle glycogen in either castrated or adrenalectomized animals to a level even above that found in unoperated controls. Furthermore, injections of testosterone into normal rats led to muscle glycogen values higher than those in the controls. His results, if they should be confirmed, would constitute the first clear evidence for the participation of the testicular hormone in carbohydrate metabolism in skeletal muscle. Previous attempts to link the secretion of the testis with general metabolic processes have been, as Koch[2] has pointed out, inconclusive.

[1] Schumann, H., *Z. f. d. ges. exp. Med.*, 1938, **103**, 117; *Ibid.*, 1939, **105**, 577; *Pflüger's Arch. f. d. ges. Physiol.*, 1940, **243**, 686; *Ibid.*, 1940, **243**, 695; *Klin. Wchnschr.*, 1940, **1**, 364.

[2] Koch, F. C., *Physiol. Rev.*, 1937, **17**, 153.

TABLE I.

Operation	Treatment	Glycogen concentration: No. of animals	Glycogen concentration: Avg muscle glycogen, mg %	Glycogen concentration: Difference from controls, mg %	Seminal vesicle, mg per 100 g body wt	Max. tension: No. of animals	Max. tension: Avg g/g
None	None	29	578		385	13	1674
"	Testosterone	13	616	38 ± 23.7*	701	7	1806
"	Desoxycorticosterone acetate	5	546	32	392	9	1591
Castration	None	36	531	47 ± 19.7	7	17	1576
"	Testosterone	26	613	35 ± 25.0	483	15	1562
"	Desoxycorticosterone acetate	7	530	48	6	6	1493
Adrenalectomy	None	16	422	156 ± 17.7	362	8	1498
"	Testosterone	20	446	132 ± 18.4	857	8	1532
"	Desoxycorticosterone acetate	7	453	125	307	8	1549

* Standard error.

It was therefore thought worthwhile to repeat some of these experiments, especially in view of the following factors in Schumann's report: 1. The normal rat muscle glycogen values reported were unusually low, about 400 mg %. 2. The rats were killed by a blow on the head, a procedure which gives inconsistent tissue glycogen values.[3] 3. The conclusions were based on only 5 rabbits, and an unstated number of rats.

Procedure. Male rats of the Sprague-Dawley strain, on a constant diet of Purina dog chow, were divided into 9 groups as shown in Table I. The animals were not fasted, but were all sacrificed about midday. Castration was performed at an average age of about 40 days; the animals were sacrificed at about 100 days of age. Three dosage levels of testosterone were used: 1. subcutaneous implants of 5-mg pellets of methyl testosterone* 2 weeks before the muscle was analyzed; 2. subcutaneous injections of 0.5 mg of testosterone propionate in 0.1 cc of sesame oil daily for one week; 3. similar injections, 1 mg daily for 3 weeks. These amounts are in excess of those shown by Greene *et al.*[4] to be adequate for maintenance of the accessory genitalia in the castrated male rat. Since there was no difference in the muscle glycogen values with the 3 levels of testosterone dosage, they have been combined for the purposes of this report (Table I). At autopsy, the seminal vesicles were dissected out and weighed to gauge the adequacy of the testosterone treatment. Desoxycorticosterone acetate was administered to some of the animals, in the form of 5-mg subcutaneous pellets, 2 weeks before sacrifice.

Bilateral adrenalectomy was performed in one stage by the usual dorsolateral approach, and the animals sacrificed 5 days later. It was found that the untreated adrenalectomized animals had lost, on the average, 13.9% of their body weight in the 5 days. Those treated with testosterone lost 15.9%, while those treated with desoxycorticosterone acetate gained a little more than 1% in weight. Gaunt *et al.* have also reported testosterone to be non-beneficial for adrenalectomized ferrets[5] and rats.[6]

For glycogen analysis, the right gastrocnemius was dissected out under nembutal anesthesia, and immediately dropped into ice cold 30% potassium hydroxide. The glucose content of the acid hydrolysate was determined by the Folin-Wu procedure, with reading in a Klett-Summerson photoelectric colorimeter. In some of the animals, maximum tension developed by the left gastrocnemius was measured by a method previously described.[7]

Conclusions. Schumann's results are not

[3] Cori, C. F., *Physiol. Rev.*, 1931, **11**, 143.

* Methyl testosterone and desoxycorticosterone acetate were obtained through the courtesy of Dr. Irwin Schwenk, Schering Corporation.

[4] Greene, R. R., Burrill, M. W., Oppenheimer, E., and Nelson, D., *Endocrinology*, 1942, **30**, 734.

[5] Gaunt, R., and Hays, H. W., *Am. J. Physiol.*, 1938, **124**, 767.

[6] Gaunt, R., Nelson, W. O., and Loomis, E., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 319.

[7] Winter, C. A., and Knowlton, G. C., *Am. J. Physiol.*, 1940, **131**, 465.

confirmed. From the data in Table I, it appears that: 1. Castration did not significantly alter muscle glycogen concentration. 2. Neither testosterone nor desoxycorticosterone, in the dosages used, affected the concentration of glycogen in the gastrocnemius muscle of the intact or the castrated male rat. 3. Neither testosterone nor desoxycorticosterone restored to normal the low muscle glycogen levels found in adrenal insufficiency. This finding, with regard to testosterone, though contrary to that of Schumann, is in agreement with the report of Gaunt *et al.*[8] 4. None of the experimental procedures led to any marked change in the maximum tension developed by the muscle.

[8] Gaunt, R., Remington, J. W., and Edelmann, A., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 429.

14474

Effects of Different Intakes of B-Complex Vitamins upon Neuromuscular Regeneration.*

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The frequency with which reports have appeared concerning the occurrence of peripheral nerve degeneration in various vitamin B deficiencies prompted us to investigate the role of vitamin B-complex in neuromuscular regeneration. This was done by making a comparative study of the rates of regeneration in animals subsisting on different levels of vitamin B-complex.

Experimental. The experiments were carried out on the gastrocnemius muscles and tibial nerves of albino rats. The animals were matched as to age, initial body weight and sex. Complete denervation of the gastrocnemius muscle of one limb was produced by crushing the tibial nerve. The non-denervated muscle and nerve of the contralateral limb served as a control. At specified times after operation measurements were made concerning the strength and weight of the regenerating and control muscles. The muscle strength was determined by measuring the maximum isometric tension which developed in response to volleys of adequate stimuli applied directly to the muscle and to its motor nerve. The technics that were employed for denervation and strength measurements have been described in detail elsewhere.[1] The quantity of functional reinnervation was estimated from the ratios of tension responses to direct muscle stimulation and to motor nerve activation. The tension responses of control non-denervated muscles to nerve stimulation were of the same magnitude as those elicited by direct muscle activation; while in the case of muscles undergoing reinnervation and regeneration the former was only a fraction of the latter.

In our studies the following basal ration has been used: sucrose 74%, purified casein 18%, salt mixture 3% and corn oil 5%. Each animal received in addition 2 mg alpha-tocopherol and 2 drops of halibut liver oil per week. The "control" group received the basal diet supplemented with 8% yeast. The "B-deficient" group received the basal ration without yeast or other B supplements. The diet designated as "B-excess" was prepared by adding to the basal diet 8% yeast and sufficient choline, calcium pantothenate, thiamine, pyridoxine, riboflavin, and rice bran concentrate (Vitab) to provide a ten-fold increase in the calculated vitamin B complex content of the diet. In view of the fact that

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[1] Hines, H. M., Thomson, J. D., and Lazere, B., *Am. J. Physiol.*, 1942, **137**, 527.

TABLE I.

A Summary of Average Values Together with Standard Errors for Normal and Regenerating Gastrocnemius Muscles of Rats Subsisting on Different Levels of B-Complex Vitamins.

Dietary condition	No. of rats	% change in body wt	Days after lesion	Gms tension per g muscle when activated through Nerve: Exp.*	Nerve: Control	Muscle: Exp.*	Muscle: Control	Relative strength of denervated muscles when activated through Nerve	Relative strength of denervated muscles when activated through Muscle	Relative weight of regenerating muscle†
Control	26	+14	21	722 ± 80	1847 ± 14	1425 ± 40	1905 ± 47	27.1 ± 2.2	50.1 ± 1.7	67.3 ± 1.3
B-deficient	20	−26	21	479 ± 46	1496 ± 64	1057 ± 47	1559 ± 59	22.3 ± 3.1	42.2 ± 1.7	64.1 ± 1.0
B-excess	12	+15	21	724 ± 60	1988 ± 74	1395 ± 36	2069 ± 31	27.5 ± 2.9	44.2 ± 1.2	65.3 ± 0.9
Fast	11	−33	21	463 ± 25	1208 ± 103	809 ± 30	1277 ± 116	32.8 ± 3.2	48.9 ± 2.8	65.1 ± 2.4
Fast + B	13	−26	21	528 ± 38	1289 ± 64	925 ± 43	1387 ± 60	29.4 ± 2.8	46.4 ± 2.0	69.2 ± 2.6
1% yeast	9	+6	21	583 ± 40	1506 ± 78	889 ± 64	1703 ± 88	25.6 ± 3.4	39.2 ± 4.2	62.3 ± 1.0
Control	15	+20	42	1148 ± 78	1608 ± 30	1295 ± 44	1691 ± 26	66.3 ± 2.6	70.5 ± 1.8	79.7 ± 1.1
B-excess	9	+19	42	1207 ± 48	1578 ± 41	1396 ± 26	1614 ± 40	69.2 ± 2.6	75.2 ± 1.5	80.5 ± 1.6

* Exp. refers to muscles previously subjected to denervation and control to contralateral non-denervated muscles.
† Expressed as % of that found in contralateral control.

animals on vitamin B-deficient diets lose considerable amounts of body weight, it seemed desirable to conduct control experiments in which comparable weight losses were brought about by total food withdrawal and by food withdrawal with provisions for supplying the required B vitamins. Accordingly one group of animals was subjected to the standard denervation operation and deprived of all food for the last 11 days of the period allowed for regeneration. Another group designated as "fast + B" was treated as above except that care was taken to provide the animals with twice their usual daily amount of B-group vitamins. An additional group was placed on the basal diet plus 1% yeast. They were allowed to subsist on this low vitamin B diet for 2 weeks before operation and then for an additional period of 3 weeks before tests were made as to the state of regeneration. Because of anorexia and the resulting loss of body weight, it was deemed advisable to terminate the regeneration studies with B-free diets after 21 days.

Results. The extent of muscle reinnervation and regeneration, as estimated from comparisons of the strength and weight of regenerating muscles to that of their contralateral controls, was but little affected by the levels of B-complex intakes (Table I). This was true for the response to motor nerve stimulation as well as to direct muscle activation. The strength per unit weight of control and regenerating muscles in animals subsisting on B-deficient diets was somewhat inferior to that of comparable muscles in animals on adequate vitamin intakes. However, the fact that even lower values were found for the strength of regenerating and non-denervated muscles of animals subjected to acute inanition and to food withdrawal with provisions for vitamin B intakes suggests that the neuromuscular weakness was due more to the effects of acute inanition than to the absence of any specific vitamin effect. It is not intended to imply that the B-vitamins are non-essential for optimum neuromuscular regeneration because it is probable that some amounts were available to our animals from bacterial and storage sources. It is apparent, however, that animals can be subjected to an acute vitamin B-complex withdrawal for a considerable period of time and show signs of B deficiency without exhibiting slower rates of regeneration.

Conclusions. Neuromuscular regeneration was not appreciably affected by acute vitamin B-complex withdrawal. Excess intakes of these vitamins failed to enhance reinnervation and regeneration. The neuromuscular weakness of regenerating and non-denervated muscles of animals on B-deficient diets was of the order of that found in animals subjected to either acute inanition or food withdrawal and B supplements.

14475

An Irregular Agglutinin and Erythroblastosis Fetalis* (Hemolytic Disease of the Newborn).

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The importance of iso-immunization, *i.e.*, immunization within the same species, in the etiology of erythroblastosis fetalis has been demonstrated.[1-7] Race and co-workers[8] state that in one of 10 pregnancies, the mother is Rh-negative and the baby Rh-positive and that in one of 5 pregnancies, the mother possesses an agglutinin for the agglutinogens A or B which are present in the blood of her fetus. Despite this statement, the incidence of clinical erythroblastosis fetalis is much less than would be expected if circulating agglutinins were capable of passing the placental barrier in all cases of iso-immunization.

In support of the theory of iso-immunization, Levine and co-workers[5] estimated that more than 90% of the mothers of erythroblastotic babies were Rh-negative in contrast to the 15% of Rh-negative individuals in a random sample of the population. The observation that 10% of women bearing children afflicted with erythroblastosis fetalis were Rh-positive suggested that factors other than Rh were involved in the etiology of this condition. Javert[7] reported a case of erythroblastosis fetalis in which the mother was Rh-positive and the father Rh-negative. An atypical agglutinogen was present in the erythrocytes of the father and of the affected fourth-born child, and absent from the cells of the mother. The specific agglutinin was present in the mother's serum. Levine tested the blood of the first-born child and found it to be Rh-positive, and lacking the agglutinogen for the atypical agglutinin present in the mother's serum. This agglutinin was designated anti-Hr by Levine.[7] Wiener[9] stated that the serum Levine termed anti-Hr agglutinated all Rh-negative and those of the Rh-positive erythrocytes which were not agglutinated by a special variety of anti-Rh serum which detected only 70% Rh-positives in a random sample-group. Therefore, he considered this reaction to be analogous to that observed in some individuals of the sub-group A_1, in whose serum the erythrocytes of group O and sub-group A_2 are agglutinated.

Race and co-workers[8] found six of a series of 50 mothers bearing erythroblastotic infants to be Rh-positive. The husbands of only 2 of these 6 subjects were available for examination, and these were found to be Rh-negative. In another observation these authors examined the fathers of 2 erythroblastotic children, borne by Rh-positive mothers, and found one father to be Rh-negative and the other to be Rh-positive. As stated by Race: "In this last case the serum of the mother (group O Rh-positive) contained a very powerful antibody more active at 37°C than at room temperature. This serum gave clear-cut positive reactions with the red cells of 80% of a large number of unselected group O persons, including her erythroblastotic child (group O Rh-positive). When tested against group O Rh-negative

* Supported by a grant from the Columbia Foundation.

1 Levine, P., Katzin, E. M., and Burnham, L., *J. A. M. A.*, 1941, **116**, 825.

2 Levine, P., and Polayes, S. H., *Ann. Int. Med.*, 1941, **14**, 1903.

3 Burnham, L., *Am. J. Obs. and Gyn.*, 1941, **42**, 389.

4 Levine, P., Vogel, P., Katzin, E. M., and Burnham, L., *Science*, 1941, **94**, 371.

5 Levine, P., Burnham, L., Katzin, E. M., and Vogel, P., *Am. J. Obs. and Gyn.*, 1941, **42**, 925.

6 Levine, P., *Am. J. Clin. Path.*, 1941, **11**, 898.

7 Javert, C. T., *Am. J. Obs. and Gyn.*, 1942, **43**, 921.

8 Race, R. R., Taylor, G. L., Cappell, D. F., and McFarlane, M. N., *Brit. Med. J.*, 1943, **2**, 289.

9 Wiener, A. S., *Blood Groups and Transfusions*, 1943, Ed. 3, C. C. Thomas, Springfield, Illinois.

bloods it was found to agglutinate strongly a series of 50 consecutive samples. After removal of the anti-A and anti-B iso-antibodies the serum still agglutinated the husband's cells (group A_1 Rh-positive). This antibody is not specific for any of the blood-cell antigens A, B, O, M, N, P, Rh, but it is suspected that it is similar to the irregular agglutinin found by Levine, Javert, and Katzin (see Levine, 1941†) in the blood of an Rh-positive mother with Rh-negative husband and an erythroblastotic child, and named by them "anti-Hr."

Gallagher and co-workers[10] observed that 9 of 21 mothers, who bore infants presenting jaundice, were Rh-positive. Potter and co-workers[11] reported an instance of erythroblastosis fetalis which occurred in a child borne of Rh-negative parents.

We take this opportunity to report our findings of an irregular agglutinin of high titer in the serum of a woman who gave birth to an erythroblastotic infant.

Case. B.B. A multiparous white woman, aged 26 years (Wassermann and Kahn tests negative) gave the following obstetric history: a spontaneous miscarriage at one month (January, 1939) and 3 induced abortions, the first at 1½ months (April, 1939), the second at one month (July, 1939), and the third at one month (February, 1941). Following curettage she was given two 500 cc transfusions of citrated whole blood (Rh factor unknown) without reaction. On January 30, 1942, she gave birth to an apparently normal full-term, living female child, who on the tenth postnatal day was found to have a hemoglobin content of 45%. No nucleated red blood corpuscles were seen. A diagnosis of anemia of the newborn was made. On 9/27/43 she delivered a living female child, weight 3480 g. The baby was weak and markedly jaundiced. The blood count showed 81% nucleated red cells and a diagnosis of erythroblastosis fetalis was made. The infant was placed in an oxygen tent and received 3 transfusions, totalling 200 cc of compatible Rh-negative blood. It died 9/29/43.

Determinations of the Rh factor were made following the birth of this child. The mother was found to belong to group A_1B, type N, Rh-positive, and the father to group O, type N, Rh-negative. The surviving child, as well as the erythroblastotic child, belonged to group B, Rh-positive. An irregular agglutinin of high titer (1:64) active at 37°C and not identifiable by the A, B, O, M, N, P, or Rh agglutinogen, was present in the serum of the mother. When tested against a series of 50 samples of blood, it agglutinated 16 samples of Rh-negative cells more strongly than the 34 Rh-positive cells. The agglutinogen counterpart of this agglutinin was present in the red blood corpuscles of her husband and of the 2 children, but was absent from her own cells. The immunization of the mother may have been produced by the aborted fetuses which could have possessed the specific agglutinogens present in the erythrocytes of the husband and/or which conceivably might also have been present in the blood which the mother received by transfusion.

Summary. An irregular agglutinin found in the serum of an Rh-positive mother of an erythroblastotic child sired by an Rh-negative father is described in this communication. This agglutinin differs‡ from that designated as anti-Hr by Levine, because it agglutinated all Rh-positive as well as Rh-negative blood samples.

† Levine, P. (1941) in *Year-book of Pathology and Immunology*, p. 509, Chicago.

[10] Gallagher, F. W., Danis, P. G., and Jones, L. R., *J. Ped.*, 1943, **22**, 171.

[11] Potter, E. L., Davidsohn, I., and Crunden, A. B., *Am. J. Obs. and Gyn.*, 1943, **45**, 254.

‡ In the case reported by Race, both parents were Rh-positive.

14476

Effectiveness of Urea and of Synthetic Detergents in Reducing Activity of Human Dental Caries.*

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It is generally accepted that dental caries is initiated by the decalcifying action of acids which are produced locally on tooth surfaces by plaques of bacteria. Several methods proposed for the control of caries have been directed toward decreasing this acidity by means of the local application of chemical agents which either reduce production of the acids or neutralize them. It has been shown by one of us that experimental dental caries in the rat can be markedly reduced by adding small quantities of fluoride or iodoacetate to the food.[1] These compounds probably produce this effect by inhibiting the activity of enzymes involved in the production of acid by the bacteria on the teeth. Since both of these substances appeared to us too toxic for prolonged use in the prophylaxis of human dental caries, a search was made for less toxic compounds with similar local action.

Certain synthetic detergents have been shown to be powerful inhibitors of glycolysis in suspensions of oral bacteria.[2,3] In suitable concentrations, they inhibit partially the production of acid in intact dental plaques.[4,5,6] It has also been shown that concentrated solutions of urea exert a double effect on the dental plaques. Urea reacts with the bacterial urease to release sufficient ammonium carbonate to render the plaque alkaline;[7] also in 40% to 50% solution, urea markedly inhibits the production of acid by the micro-organisms in the plaques.[6,8] This paper presents the results of observations made to evaluate the effectiveness of a cationic synthetic detergent, and of urea in the control of human dental caries.

Procedure. Young individuals with marked caries activity were selected from patients who had been examined and treated in the Zoller Memorial Dental Clinic. These individnals had been taught the proper use of the tooth brush for home care of the mouth. They were observed during a control period of about 18 months. Full mouth and bite-wing X-rays, as well as complete clinical mouth examinations were obtained several times during this period.

A group of these patients was given a flavored solution of 1:1000 alkyldimethylbenzyl ammonium chloride (Zephiran).† Another group was given a flavored, saturated solution of urea.‡ The patients were instructed to

* This investigation was aided in part by a grant from the Squibb Institute for Medical Research.

1 Miller, B. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 389.

2 Miller, B. F., Baker, Zelma, and Harrison, R. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 705.

3 Baker, Zelma, Harrison, R. W., and Miller, B. F., *J. Exp. Med.*, 1941, **73**, 241.

4 Miller, B. F., Muntz, J. A., and Bradel, S., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 104.

5 Stephan, R. M., and Miller, B. F., *J. Dent. Res.*, 1943, **22**, 53.

6 Muntz, J. A., and Miller, B. F., *J. Dent. Res.*, 1943, **22**, 73.

7 Stephan, R. M., *Science*, 1940, **92**, 578.

8 Stephan, R. M., *J. Dent. Res.*, 1943, **22**, 63.

† We have referred in the title of this paper to the use of "synthetic detergents" to stress our belief that a number of cationic detergents would be as satisfactory as Zephiran. At the present time we have a number of subjects using other cationic detergents as well as Zephiran.

Zephiran solution:
Zephiran (Alba Pharmaceutical Co.) 10 cc of 10% aqueous solution
Oil of wintergreen 0.5 "
Distilled water to 1000 "

‡ *Urea solution:*

	g
Urea (DuPont)	45.0
Distilled water	47.2
Alcohol (95% ethyl)	7.0
Menthol	0.1
Saccharin	0.3
Gomagel (thickening agent made by Glyco Products Co., 230 King Street, Brooklyn, N.Y.)	0.4

TABLE I.
Effect of Urea and Zephiran on Dental Caries Activity.

Case and age*	Period	Months of observation	No. intact surfaces at start†	No. new carious surfaces	No. new carious surfaces per 100 intact surfaces per yr
		Urea Cases.			
E.B.	Control	19	97	20	13.0
15	Urea	24	78	4	2.6
O.B.	Control	19	115	16	8.8
14	Urea	21	107	1	0.5
R.D.	Control	16	75	29	29.0
15	Urea	16	43	0	0.0
J.J.	Control	34	63	44	25.0
25	Urea	26	26	0	0.0
A.M.	Control	18	118	16	9.0
16	Urea	25	90	0	0.0
E.T.	Control	24	118	27	11.0
14	Urea	27	92	3	1.4
		Zephiran Cases.			
C.B.	Control	16	90	18	15.0
12	Zephiran	21	90	8	5.1
R.B.	Control	24	102	15	7.3
20	Zephiran	18	91	1	0.7
T.C.	Control	13	93	12	12.0
13	Zephiran	17	92	7	5.3
G.H.	Control	18	97	9	6.1
16	Zephiran	16	88	1	0.8
C.H.	Control	19	106	14	8.3
13	Zephiran	24	96	6	3.1
E.R.	Control	24	101	36	18.0
13	Zephiran	25	90	6	3.2
		Duplicate Control Cases (No change in dentrifice).			
W.G.	Control	21	108	14	7.4
14	Duplicate	21	102	11	6.2
A.H.	Control	19	122	16	8.3
15	Duplicate	20	99	14	8.5
R.M.	Control	14	116	9	6.7
13	Duplicate	16	104	10	7.2
J.B.	Control	19	93	6	4.1
12	Duplicate	19	95	6	4.0
D.A.	Control	18	114	12	7.0
16	Duplicate	19	102	15	8.4
M.B.	Control	18	81	12	9.8
20	Duplicate	19	66	10	9.6

* Age in years at start of test period.

† Based on counting the mesial, buccal, distal, and lingual surfaces for anterior teeth, and the occlusal surface in addition to these for posterior teeth. Carious and filled tooth surfaces not counted.

apply these solutions with a toothbrush to all accessible tooth surfaces for a period of 4 minutes either before or immediately after breakfast and supper. They were told to make no changes in their dietary habits. Clinical and roentgenological mouth examinations were made at 3 to 4 month intervals during the experimental period and a careful search was made for the development of new carious lesions. The numbers of intact tooth surfaces which became carious during the period in which the solutions were used was compared with the numbers which became carious during the preceding control period.

Results. In this paper observations on patients who had used the urea or Zephiran solutions for a period of 16 months or longer and who had had an equivalent control period are reported. It is felt that as long a period of time as this is essential in comparing caries activity rates. In order to secure a quantitative expression of caries rates, we have calculated for each individual the number of new carious lesions which develop per 100 intact tooth surfaces per year.

The degree of caries susceptibility of indi-

vidual tooth surfaces is variable; therefore we have sought to determine whether in young individuals as selected for this study the caries rate would tend to be the same in 2 consecutive periods of observation when no changes were made in the agent used in home care of the teeth. For this purpose we have included a group of patients who after the control period continued the use of their regular dentifrice. The results from 6 patients who used urea, 6 patients who used Zephiran, and 6 patients who continued use of their regular dentifrice are listed in the Table I.

In the first group of 6 subjects the number of new carious lesions appearing during use of the urea solution was only 8 whereas in the preceding control periods 152 new lesions had developed. Individually there was a reduction of from 80 to 100% in the original caries rates which resulted from the use of urea. This represents a great decrease in caries activity.

In the second group of 6 subjects 29 new carious lesions developed while Zephiran solution was being used, whereas in their control periods they had developed 104 new carious lesions. Individually there was a reduction of from 56 to 90% in the original caries rates which resulted from use of the Zephiran solution. This indicates a considerable decrease in caries activity.

In the third group of 6 subjects 71 new carious lesions appeared during the second period (duplicate control) in which no change was made in the dentrifice, and in the preceding control period 69 new lesions had formed. It is apparent that no significant reduction in the caries rate for these individuals occurred during the second period of study when the other subjects were using urea or Zephiran.

An attempt was made to determine the effect of the urea and Zephiran on the progress of carious lesions. A comparison was made of the size of individual lesions as seen in X-rays taken at the beginning, during, and at the end of the study periods. In the urea cases a significant number of the lesions appeared arrested while progress in most of the others was slower than in the control period. In 2 of the Zephiran cases progress in carious lesions appeared to be reduced but in the other 4 cases progress of lesions was about the same as in the control period. However, due to the placing of fillings, and also to inequalities of density and angulation of the X-rays, no exact quantitative expression of caries arrest could be obtained.

Discussion. These findings indicate that the rate of formation of new carious lesions can be decreased by the use of certain solutions applied repeatedly to the teeth. Presumably these solutions produce their beneficial effects by reducing the acidity of the dental plaques.[4,5,6,7,8] It is interesting that urea, though less effective *in vitro* as an inhibitor of acid production by suspensions of bacteria than Zephiran, is more effective in these clinical tests. This relationship correlates well with our previous studies showing the greater ability of urea to diffuse into thick dental plaques and inhibit acid formation.[6,8] In addition, the neutralizing power of urea by production of ammonium carbonate probably contributes to its effectiveness.

There were no serious difficulties in getting patients to use the solutions in brushing their teeth. Some subjects using Zephiran developed a thin layer of brown material on the teeth. This could be largely removed by daily brushing with an ordinary dental paste or powder which contains abrasive material. Some patients when first employing the urea solution produced an abrasion of the gingivæ by scrubbing with the tooth brush. This gingival abrasion disappeared when these patients brushed their teeth with the correct "sweeping" technic. The chief difficulty has been in securing daily applications of the solutions for the prescribed length of time. The possibility of lessened caries incidence has not always proved an adequate incentive for this, even though the tastes of the solutions have been generally satisfactory§ and the times for tooth brushing have been arranged for convenience.

The number of cases presented here is small

§ The reaction to the taste of the urea or Zephiran solutions appears to be highly individualized. Some persons find the taste quite pleasant from the beginning, others become accustomed to the taste in a few days, and a few find that the taste is disagreeable.

because of difficulties encountered in maintaining adequate contact with patients over a period of 3 to 4 years. However, we feel that the cases reported have been sufficiently well controlled to indicate that the regular use of the concentrated solution of urea can be very useful in the prophylaxis of dental caries.

Summary. Solutions of urea and of synthetic detergents have been employed as dentifrices for approximately 2 years in a small group of patients suffering from severe dental caries. The use of these compounds, especially the urea, resulted in a marked decrease in the incidence of new lesions, and the urea solution also retarded the progress of caries in lesions already present.

The authors wish to express their appreciation to the members of the Walter G. Zoller Memorial Dental Clinic staff for their cooperation in this study.

14477 P

Protective Effect of Vaccination Against Induced Influenza A.*

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During the past year from December, 1942, to May, 1943, rather extensive studies were conducted for the purpose of determining the value of subcutaneous vaccination with inactivated influenza virus against the natural disease in man. The anticipated outbreak of influenza did not occur but the opportunity to test the resistance of certain of the vaccinated individuals to induced infection presented itself.

The vaccine represented the allantoic fluid of fertile hen's eggs infected 48 hours previously with either the PR8 strain of influenza virus, Type A, or the Lee strain, Type B. The virus was concentrated by the procedure of Francis and Salk,[1] inactivated with formalin 1:2000, and bottled and tested for sterility by the approved procedures for biological products. Phenyl mercuric nitrate, 1:100,000 was added for bacteriostatic purposes. The final vaccine was of such strength that 1.0 cc represented 5.0 cc of the original Type A fluid and 5.0 cc of Type B fluid.

The subjects were 102 physically active male residents of one ward of the Ypsilanti State Hospital, Ypsilanti, Michigan. On December 21, 1942, 45 received 1.0 cc of the vaccine subcutaneously. On April 21, 1943, 17 of these men again received 1.0 cc of the vaccine as did 21 men not previously vaccinated. There remained 28 men who had been vaccinated 4 months earlier and 36 individuals who had not been vaccinated at any time.

On May 4, 1943, 13 days after the last administration of vaccine, the entire group received an intranasal spray of the Baum‡ strain of Type A virus in allantoic fluid. The material was sprayed into the nostrils from a nebulizer with such fineness that approximately 0.5 cc was delivered in 4 minutes. The subjects were then strictly segregated from the rest of the institution. Oral tempera-

* These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

This study was also aided by a grant from the International Health Division of the Rockefeller Foundation.

† Fellow in the Medical Sciences of the National Research Council.

1 Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

‡ The Baum strain was recovered from a patient during the outbreak of 1940-41. Although it is clearly a strain of Type A it is not antigenically identical with the PR8 strain used in the vaccine.

TABLE I.
Effect of Subcutaneous Vaccination upon Febrile Response of Human Subjects to Induced Infection with Influenza Virus, Type A.

Vaccination record	No. of subjects	Highest temperature <99 No.	<99 %	99+ No.	99+ %	100+ No.	100+ %	101+ No.	101+ %	102+ No.	102+ %
1. Unvaccinated	36	7	19	29	80	18	50	9	25	4	11
2. 4½ mos. before	28	8	28	20	71	9	32	3	10	1	3
3. 4½ mos. and 2 wks. before	17	6	35	11	64	3	17	0	0	0	0
4. 2 wks. before	21	7	33	14	66	3	14	0	0	0	0

tures had been taken 2 days before exposure and at least twice daily thereafter. Observations for signs and symptoms of infection were maintained. Materials for other clinical and serological studies were collected.

In a proportion of the subjects a clinical infection resembling a mild form of epidemic influenza occurred. The incubation period was generally not longer than 24 hours. The onset was accompanied by chills or chilliness, aches, prostration, and minor respiratory complaints. Fever as high as 103°F was noted, to a great extent paralleling the severity of symptoms. In most instances it did not persist longer than 48 hours.

When the responses to inhalation of virus of the different groups of subjects were analyzed it was found that distinct and significant differences obtained. This is clearly reflected in the febrile reactions presented in Table I. Temperatures of 100° or more were observed in 50% of the controls, in 32% of those vaccinated 4½ months before exposure, in 14.3% of those vaccinated 2 weeks before and in 17.6% of those vaccinated both 4½ months and 2 weeks before; the incidence of fever in the 2 groups, totalling 38 individuals, who thus had been vaccinated 2 weeks before infection, was 15.8%. Moreover, none of the latter had temperatures higher than 100.6° while 9 of the control group and 3 of the men vaccinated 4½ months earlier had fever of 101° or higher.

The results indicate that the vaccine employed induced a state of resistance, manifested in reduced febrile responses to intranasal infection with influenza virus, Type A. This effect was most evident in those vaccinated 2 weeks before exposure. After 4½ months the protective influence of vaccination had declined considerably, just as has been found to be the case 4 months after infection with influenza virus, Type B.[2] There was no indication that 2 inoculations 4 months apart had any additive effect. While the present evidence agrees with that of Henle, Henle, and Stokes[3] in showing that subcutaneous vaccination clearly exerts a beneficial influence against induced infection with influenza virus, Type A, it differs in demonstrating that the effective immunity so established was considerably reduced after an interval of 4 months. This difference may be related to the fact that, as indicated by the higher incidence rate in controls, the present test was more severe.

A detailed report will be published later.

2 Francis, T., Jr., Pearson, H. E., Salk, J. E., and Brown, Philip N., *Am. J. Pub. Health*, 1944 (April).

3 Henle, W., Henle, G., and Stokes, J., Jr., *J. Immunology*, 1943, **46**, 163.

14478 P

Protective Effect of Vaccination Against Induced Influenza B.*

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In the previous paper[1] evidence was presented to show that subcutaneous inoculation of vaccine containing concentrated and inactivated influenza virus, Types A and B, was followed by an increased resistance of human individuals, as measured by the febrile response, to a subsequent intranasal spray of active Type A influenza virus. The present report deals with the results of a similar study of the effect of subcutaneous vaccination upon resistance of another group of individuals in the same institution to infection with influenza virus, Type B.

The vaccine was the same material employed in the study with influenza A. The subjects were 96 physically active male residents of another ward of the Ypsilanti State Hospital, Ypsilanti, Michigan.

On December 21, 1942, 46 of the men received 1.0 cc of the vaccine subcutaneously. On April 13, 1943, 19 of the same individuals received a second injection of 1.0 cc of the vaccine and 23 men not previously vaccinated now received 1.0 cc of the vaccine. There remained 27 men vaccinated in December but not since, and 23 control subjects who had not at any time received vaccine.

On May 10, 1943, 27 days after the administration of vaccine to 2 of the groups, all 96 men received the Lee strain of influenza B virus by intranasal inhalation. The virus from the allantoic fluid of the infected hen's egg had been concentrated[2] tenfold in physiological salt solution. The material was sprayed for 5 minutes under 10 lb air pressure from a nebulizer so as to deliver approximately 0.5 cc of the virus. Oral temperatures had been recorded twice prior to the inhalation of the virus and were continued twice daily thereafter. Records were kept of signs or symptoms of illness. Material for other clinical and serological studies was collected.

In those individuals in whom evidence of infection was discerned, the incubation period was less than 24 hours and the febrile period was in only one instance longer than one day. The physical evidence of illness was also mild with chilliness, aches, cough, and malaise. The highest temperature was 102.6°F No significant complications were noted. The distribution of febrile reactions in the different groups is detailed in Table I.

In the unvaccinated group, 11 or 41%, of the 27 individuals had temperatures of 100° or more and 6, or 22%, had temperatures of 101° or above. In the 69 vaccinated individuals, 7, or 10%, had temperatures between 100 and 100.9° while none exceeded that range. There was no significant difference in the responses of the groups vaccinated 4½ months before, 4 weeks before or vaccinated twice before exposure. In this respect the results differ from those obtained with influenza virus, Type A, where the group vaccinated 4 months prior to exposure appeared less uniformly resistant than those vaccinated 2 weeks before virus was administered.

The results demonstrate that subcutaneous injection of vaccine of concentrated and inactivated influenza virus, Types A and B, is capable of exerting a protective influence, as measured by the febrile response, against

* These investigations were aided through the Commission on Influenza, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

This study was also aided by a grant from the International Health Division of the Rockefeller Foundation.

† Fellow in the Medical Sciences of the National Research Council.

1 Francis, Thomas, Jr., Salk, J. E., Pearson, H. E., and Brown, Philip N., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 104.

2 Francis, Thomas, Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

TABLE I.
Effect of Subcutaneous Vaccination upon Febrile Response of Human Subjects to Induced Infection with Influenza Virus, Type B.

Vaccination record	No. of subjects	Highest Temperature <99 No.	%	99+ No.	%	100+ No.	%	101+ No.	%	102+ No.	%
1. Unvaccinated	27	5	19	22	81	11	41	6	22	2	7
2. 4½ mos. before	27	12	44	15	56	2	7	0	0	0	0
3. 4½ mos. and 4 wks. before	19	4	21	15	79	2	11	0	0	0	0
4. 4 wks. before	23	12	52	11	48	3	13	0	0	0	0

induced infection with Type B influenza virus for at least one to 4 months. These data together with those in the preceding paper show that the same mixed vaccine induced an increased resistance of human individuals to infection with virus of Type A or Type B.

Further details will be reported subsequently.

14479 P

Effect of Biotin Deficiency on the Course of *Plasmodium lophuræ* Infection in Chicks.

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It has generally been assumed that malnutrition decreases the resistance of the organism to malaria. The first experimental evidence to support this view is Trager's[1,2] recently published observation suggesting that biotin deficiency increased the severity of certain avian malaria infections. Similarly, Caldwell and György have reported that biotin deficiency will prolong *Trypanosoma lewisi* infections in the rat.[3]

Since we had found, in experiments to be reported soon, that a deficiency in certain dietary factors which have not yet been chemically characterized has a pronounced effect on the course of *Plasmodium lophuræ* infections in chicks, we were interested in determining whether or not the effect of biotin deficiency on avian malaria was specific. In this communication we are reporting experiments on the relationship of biotin deficiency to the severity of *P. lophuræ* infections which, although the diets and experimental procedures used differed from those of Trager, confirm his findings on the specific effect of biotin.

Experimental. While *P. lophuræ* infections are more severe in the duck than in the chick the latter species was chosen for the following experiments because much more is known about its nutritional requirements. Because of simplicity and convenience it was decided to produce biotin deficiency for this experiment by adding dried raw egg white to a commercial chick ration. In order to be certain that the addition of egg white proteins did not in itself influence the course of the malaria, a control diet was used in which steamed egg white replaced the raw egg white. To further demonstrate whether or not biotin was the specific factor involved, 0.0001% of crystalline biotin was added to the diet containing dried raw egg white.

Twenty-five 9-day-old S.C.W. Leghorn male chicks were set out on each of the following diets:

1 Trager, W., *Science*, 1943, **97**, 206.

2 Trager, W., *J. Exp. Med.*, 1943, **77**, 557.

3 Caldwell, F. E., and György, P., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 116.

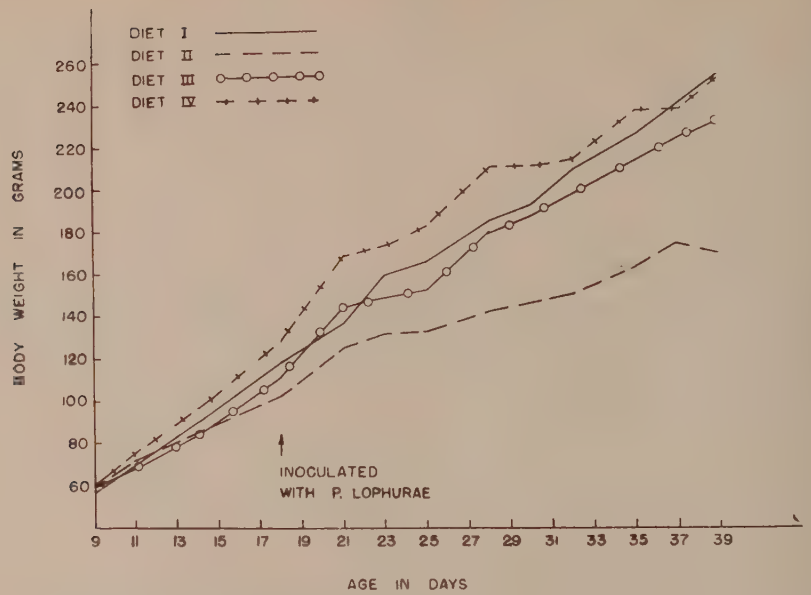

FIG. 1.

Growth curves of chicks infected with *P. lophuræ* and receiving diets adequate (I, III, IV) or deficient (II) in biotin.

TABLE I.

Percent Erythrocytes Parasitized by *P. lophuræ*.

Days after inoculation	Diets I Mean ± S.E.	II Mean ± S.E.	III Mean ± S.E.	IV Mean ± S.E.	Least significant difference*
3	0.72 ± 0.09	0.78 ± 0.07	0.53 ± 0.06	0.54 ± 0.06	NS
5	1.76 ± 0.23	3.30 ± 0.41	2.24 ± 0.38	1.64 ± 0.21	0.93
7	0.66 ± 0.30	1.87 ± 0.89	0.30 ± 0.10	0.29 ± 0.08	NS
9	0.09 ± 0.05	0.14 ± 0.10	0.02 ± 0.01	0.003	NS

* Probability = 5%; NS = differences between means are not significant.

I. Startena.*
II. 85% Startena + 15% dried raw egg white.
III. 85% Startena + 15% steamed egg white.
IV. Diet II + .0001% crystalline biotin.

The dried raw egg white was obtained on the market and the steamed egg white was prepared by exposing this material to moist heat of 100°C for one hour. Crystalline biotin was obtained through the courtesy of Dr. Folkers of Merck & Co.

After the chicks had been on the above diets for 9 days, when the growth retardation of the birds on the biotin-deficient diet (Diet II) became apparent, 15 of each diet group were inoculated intravenously with a saline suspension of 10,000,000 erythrocytes parasitized by *P. lophuræ* per 50 grams of body weight. Beginning on the third day after inoculation, blood smears were made every 48 hours and the number of parasitized erythrocytes per 10,000 erythrocytes was counted.

Results. During the course of the experiment the rate of growth of the biotin-deficient birds (Diet II) was poor (Fig. 1). Lesions on the legs and beaks, characteristic of biotin deficiency, were first noticed on the third day after inoculation. There was no difference in the growth rate and the extent of the lesions

* Trade name for a chick starting mash manufactured by the Ralston Purina Company, St. Louis, Missouri.

between the infected and the uninfected birds. None of the birds on Diets I, III, and IV showed any gross evidence of deficiency disease.

Mean parasite counts (Table I) in the biotin-deficient birds (Diet II) at the peak of their infections were approximately twice as high as those of the birds whose diets contained a presumably adequate amount of biotin (Diets (I, III, and IV). It is evident from the above results that the peak parasite counts in *P. lophuræ* infections are much greater in chicks on a diet deficient in biotin than in chicks on a diet which is identical except that it contains an adequate amount of biotin. It is not know whether the lack of biotin stimulates the multiplication of the parasites *per se,* whether it interferes with a defensive mechanism in the host, or whether both factors may be involved.

14480

Effect of Nerve Compression on Wallerian Degeneration *in Vitro.**

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Wallerian degeneration is an essential preliminary to successful nerve regeneration in that it prepares the distal stump for reinvasion by regenerating fibers. Physiological insight into its mechanism, however, lags greatly behind our descriptive acquaintance with its morphological features. The following experiments represent part of a broader study of the factors determining the character and rate of Wallerian degeneration.

It was known that when a nerve locally constricted by a ligature,[1] clamp,[2,3] or arterial cuff,[4] undergoes Wallerian degeneration, those parts of the axis cylinders lying within the compressed zone may fail to break down. This effect could be ascribed (1) to injury of the fibers, or (2) to paralysis of the mechanism of degeneration either (a) by the local ischæmia, or (b) by direct pressure action on the nerve fiber. Alternative a and b could be decided by reproducing the compression effect *in vitro, i.e.,* with the whole nerve removed from blood supply; alternative 1 and 2 by the demonstration that fibers arrested in their degeneration by compression may degenerate after decompression.

The capacity of nerve fibers to undergo typical Wallerian degeneration in a properly composed medium *in vitro* has been known.[5,6] Our own observations confirm, with certain qualifications, the earlier reports: Degeneration occurs promptly in Ringer's, Tyrode's solution, blood serum, but less regularly or not at all in NaCl solution or blood plasma. Our present experiments were carried out with fragments (*cca.* 1 cm) of rat nerves (sciatic, brachial, intercostal; 100-200 g donors), explanted into Ringer's solution in Petri dishes at 37°C, with or without compression, fixed after varying intervals sectioned longitudinally, and silver-impregnated according to Bodian.[7] Criteria of degenera-

* This work was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago; also aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

1 Cajal, S. Ramôn y, *Degeneration and Regeneration of the Nervous System,* Oxford University Press, London, 1928, Vol. I, p. 292.

2 Stroebe, H., *Beitr. z. path. Anat.,* 1893, **13**, 160.

3 Denny-Brown, D., and Brenner, C., *Arch. Neurol. and Psych.,* 1944, in press.

4 Weiss, Paul, and Davis, Hallowell, *J. Neurophysiol.,* 1943, **6**, 269.

5 Ingebrigtsen, Ragnvald, *J. Exp. Med.,* 1916, **23**, 251.

6 Nageotte, Jean, *L'Organisation de la Matière,* Paris, Félix Alcan, 1922, p. 274.

7 Bodian, David, *Anat. Rec.,* 1937, **69**, 153.

FIG. 1.

Tibial nerve with constricting arterial cuff (*a*) after 12 hours *in vitro*. Most fibers inside sleeve still intact, fibers outside vacuolated and in fragmentation. × 114.

FIG. 2.

Tibial nerve with arterial cuffs (*a*) after 24 hours *in vitro*. × 138.

(a) Constricting cuff. Many intact fibers inside of cuff; practically all fibers outside of cuff degenerated. Note the sharpness of demarcation line.

(b) Non-constricting cuff. Practically all fibers uniformly degenerated both inside and outside of cuff.

tion were the changes of the axis cylinder, progressing as follows: swelling → vacuolization → fragmentation → ovoids.

In a preliminary series, nerves were placed under 25 g glass slides and compared with unweighted nerves in the same dish. Nine nerves thus compressed showed definite delay of degeneration as compared with their controls after 18, 26, and 48 hours. That the delay was due to pressure and no other factors (oxygen lack, accumulation of metabolites, etc.), is evidenced by 3 nerves kept between slides propped on silk threads of corresponding size, which degenerated without delay. In 5 of 8 nerves compressed to only half their lengths, degeneration in the compressed end lagged behind that of the free end.

More uniform and effective application of pressure was achieved by the use of constricting sleeves of live artery (carotid or femoral) slipped over the nerve fragments as described previously.[8,3] By a suitable choice of size combinations of nerves and arteries, various degrees of constriction were obtained. Nerve parts left bare or provided with sleeves of matching size served as controls. In some cases, the sleeves were removed after varying periods of explantation as a test of the results of decompression. Samples for histological study were taken from the same preparation at different stages of its *in vitro* life.

In all cases in which constriction had been adequate, Wallerian degeneration was markedly retarded in the compressed as compared with the uncompressed portions of the same nerve sample. The effect was clearly one of

[8] Weiss, Paul, *Arch. Surg.*, 1943, **46**, 525.

TABLE I.
Reduction of Degeneration Index by Arterial Constriction of Explanted Nerves.

No.		Nerve*	Artery†	Period of explantation, hrs	Compressed Zone: Total of fibers counted	Compressed Zone: Degeneration index (i_c) %	Uncompressed Zone: Total of fibers counted	Uncompressed Zone: Degeneration index (i_u) %	Compression effect $\Delta = i_u - i_c$ %	σ_Δ %	P	Significance
1462	A	Tib	car	24	299	32	277	46	+14	4.1	<.01	+
	B	”	”	24	328	38	300	54	+16	4.0	<.01	+
	C	”	”	48	318	27	253	45	+18	4.0	<.01	+
1463	A	Per	fem	24	103	48	93	43	— 5	7.1	.48	—
	C	”	”	48	106	31	86	34	+ 3	6.8	>.50	—
1464	A	Tib	car	24	324	30	260	72	+42	4.2	<.01	+
	B	”	”	24	293	34	306	49	+15	4.0	<.01	+
	C	”	aorta	48	305	39	364	53	+14	3.9	<.01	+
	D	”	car	48	308	26	254	50	+24	4.1	<.01	+
1465	A	Per	”	24	164	47	148	70	+23	5.6	<.01	+
	B	”	fem	24	122	26	135	34	+ 8	5.7	.16	—
	D	”	”	48	89	28	127	29	+ 1	6.3	>.50	—
1469	A	Br	”	24	230	74	240	99	+25	3.2	<.01	+
	B	”	”	24	189	87	179	96	+ 9	3.5	.01	±
	C	”	”	48	244	77	297	97	+20	3.0	<.01	+
	D	”	”	48	161	92	118	97	+ 5	3.5	.16	—
1470	A‡	Br	”	24	396	52	354	92	+40	3.3	<.01	+
	B	”	”	24	226	80	195	92	+12	3.4	<.01	+
	C	”	”	24	187	89	159	92	+ 3	3.1	.32	—

* Tib = tibial; Per = peroneal; Br = brachial.
† car = carotid; fem = femoral; all arteries from 95 g donors except 1470 (150 g donor).
‡ Reproduced in Fig. 2a.

delay rather than of arrest, since the difference tended to disappear in specimens kept long enough for degeneration to run its course in both experimental and control stretches (*cca.* 48 hours). Fig. 1 shows the differential after 12 hours, Fig. 2a after 24 hours, while Fig. 2b proves the ineffectiveness of non-constricting sleeves.

As the fiber population of a nerve does not degenerate in strict synchronism, the progress of degeneration can be measured by the ratio of degenerated fibers (axon already fragmented) to preserved fibers (axon still continuous). The percentage of degenerated fibers may be referred to as the "degeneration index." In an average of 5 random sample sections of each of 19 nerves, the "degeneration index" was determined for the compressed and the uncompressed portions. Table I gives the results with statistical analysis. In 13 cases the difference between the compressed and uncompressed parts is of high statistical significance (9-42%; $P < 0.01$). Of the 6 statistically not significant cases, 4 are peroneal nerves which, owing to their smaller size, have suffered only minor constriction or none, and one is a 48-hour case too far advanced to show a differential.

Decompression experiments have demonstrated that the released fibers continue to degenerate, many apparently at a reduced rate, and evidence of their lag may still be present 24 hours later. Experiments in which explanted nerves were subjected to longitudinal stretch produced no obvious effects on degeneration.

Our results lead to the following conclusions. Pressure impairs, but does not destroy, the capacity for Wallerian degeneration. The impairment is not due to vascular interference since it can be reproduced *in vitro* in the absence of all circulation. Of the constricting and sheathing effects of an arterial sleeve, only the former affects degeneration, since a nerve segment insulated from the medium by a non-constricting artery degenerates without delay. Variations in the state of degeneration along a peripheral nerve course may be but the result of pressure fluctuations. Pressure could perhaps also account for the failure of

nerves to degenerate in plasma-clots[5] in contrast to liquid serum, as such clots develop strong contractile forces in their interior.[9]

Summary. Lateral compression of peripheral nerves delays Wallerian degeneration of the compressed zone *in vitro*.

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14481 P

Effect of Cell Growth Activating Tissue Extracts, Locally Applied, on Experimental Skin Wounds.*

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In previous communications from this laboratory[1–4] it was shown that extracts of adult tissues possess pronounced cell growth-promoting power. Moreover, the growth-promoting activity of tissue extracts was found to be completely independent of the state of proliferation of the tissue from which they are made.[5,6] Extracts of certain adult organs—heart, smooth muscle, and brain—activate the growth of fibroblast colonies *in vitro* to a greater extent than embryonic extract. The stimulating action of adult tissue extracts in cell growth is not transient. It is possible to cultivate cells indefinitely in a medium which is composed exclusively of adult plasma and extract of adult heart or adult brain.[7] The growth-promoting property of extracts of adult tissues is not species-specific. The growth of human skin epithelium can, for instance, be activated by extract of adult fowl heart to a high degree.[8] The active principle in the growth-promoting tissue extracts is probably a protein or a protein linked group.[9]

In numerous investigations[10–21] it was claimed that local application of cell growth-promoting embryonic extract accelerates the process of wound healing in man and animals. The extracts of certain adult tissues being more powerful in stimulating cell proliferation *in vitro* than embryonic extract, and moreover, much easier to obtain than the latter, the use of growth-activating adult tissue extracts in wound healing was suggested.[8] A trial of the effect of the cell growth-stimulating adult tissue extracts on the process of healing of wounds under properly controlled conditions of animal experiments appeared therefore to be desirable.

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† Working under the Hadassah Medical Organization fellowship.

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TABLE I.

The Results of Wound Healing Experiments. Each group contains rats with two symmetrically placed skin wounds, one of which was submitted to the tissue extract treatment, and the other was used as a control.

Treatment	No. of rats	Mean closure time in days	
		Exp.	Control
A. Saline adult heart extract	10	19.1	17.7
B. Alcohol precipitates of adult heart extract			
1. Powder	22	18.9	18.5
2. Ointment	14	17.1	15.4
C. Saline embryonic extracts	20	23.1	18.5

In the experiments reported here the question is examined whether adult tissue extracts and cell growth-promoting preparations therefrom, applied locally, are capable of accelerating the process of healing. In addition, the closure time of treated wounds has been compared to that of untreated wounds in the same animal.

The experiments were performed on white rats. Two circular symmetrical wounds, each about 20 mm in diameter, were produced on the back, the skin being excised down to the superficial fascia. The wounds were treated with (1) saline extracts of adult chicken hearts incorporated in an ointment base (40% saline heart extract and 60% lanolin) and (2) with alcohol precipitates of these extracts prepared according to a method previously described,[9] and applied in the form of an ointment or dry powder. (Tested in tissue cultures, the growth-promoting activity displayed by the alcohol precipitate, dissolved in Tyrode solution, is not inferior to that of the native extracts.[9])

The ointments were applied to the wounds in a 2-3 mm layer, and the wounds were covered with a gauze dressing. The control wounds were covered with the same ointment base without additional tissue extract. Powder was sprinkled on the surface of the wounds directly, and the wounds covered with gauze and protective dressing. The ointment and the powder were renewed every second day. The experimental and control wounds were measured at intervals of 48 hours. Cases of infected wounds were excluded from the tabulated data.

The experiments showed that extracts of adult heart and alcohol precipitate from these extracts do not accelerate the course of healing of treated wounds as compared with controls in the same animals. The course of all phases of the wound healing process (contraction, formation of granulated tissue, epithelization) is the same in treated as in control wounds.

In view of this result it seemed to us of interest to re-examine the findings of earlier investigators on the allegedly positive effect of embryonic extract in wound healing. 33% saline extracts of 7-day-old chicken embryos were used in these experiments. The wounds were covered with gauze saturated, in the case of the experimental wound, with embryonic extract and, in the case of the control wounds, with Ringer solution. The extract and Ringer solution were renewed daily. Our experiments showed that no acceleration of healing rate could be obtained in wounds treated with embryonic extract, as compared with untreated controls; on the contrary, in wounds treated with embryonic extract, definite inhibition of the healing process was observed.

The results of the experiments reported are summarized in Table I.

Summary. The extract of adult chicken hearts, alcohol precipitates of this extract, and extract of 7-day-old chicken embryos were applied to experimental skin wounds in rats. No acceleration of the wound healing process could be obtained with the preparations used. The period of repair for wounds treated was not shorter than for control wounds in the same animals.

14482

Increased Resistance to Anoxia After Thyroidectomy and After Treatment with Thiourea.

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Several reports have shown that removal of the thyroid increases the resistance of the rat to anoxia, presumably because this operation reduces the consumption of oxygen.[1–5]. However, some authors were unable to find differences between normal and thyroidectomized rats exposed to low oxygen pressures.[6,7] Our first investigation of this problem was made on thyroidectomized rats which had been fasted overnight to reduce their oxygen consumption to a basal value; the resistance of these rats to anoxia did not significantly differ from that of intact controls similarly fasted. Meanwhile, it was found that fasting itself affects the sensitivity to low oxygen pressures.[8,9] It was therefore necessary to expose to anoxia fasted and fed thyroidectomized rats concomitantly with similar groups of intact animals. Similar tests were carried out in animals treated with thiourea, a drug preventing the thyroid from releasing its hormone.[10,11] Since it was also desired to examine the effect of metabolic stimulations on resistance to anoxia under controlled feeding conditions, parallel experiments were carried out in animals treated with thyroxine or dinitrophenol. Both the thyroid hormone[3,12,13] and dinitrophenol[3,14] were previously reported as decreasing the resistance to a low oxygen pressure.

For the anoxia tests, the animals were placed in a continuous-flow low pressure chamber (21-24°C), where they were kept for 15 minutes at each one of the following pressures in succession: 200, 175, 150, 125, and 100 mm of mercury.[8,9] The resistance to anoxia was estimated by measuring the length of survival in minutes counting from the beginning of the experiment. For instance, a survival of 12 minutes indicates that the animal died at the pressure of 200 mm; a survival of 56 minutes shows that death occurred after 11 minutes at the pressure of 125 mm (Fig. 1). The averages for the survival of each group are reported in the tables with their standard errors; these were helpful in interpreting the results, although their significance was somewhat affected by the fact that the variations of the stimulus (pressure levels) were not continuous.

Part of a group of male rats weighing from 50 to 80 g were thyroidectomized, the rest being kept as controls. The anoxia tests were performed one month later. All the animals were left without food overnight, and then separated into 4 groups of 6 animals each. One normal and one thyroidectomized group remained unfed, while another normal and another thyroidectomized group received food for one hour. All were exposed to the low oxygen pressures 3 to 4 hours after the feeding period. The results (Table I) show that

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14 Tainter, M. L., *J. Pharm. and Exp. Ther.*, 1934, **51**, 45.

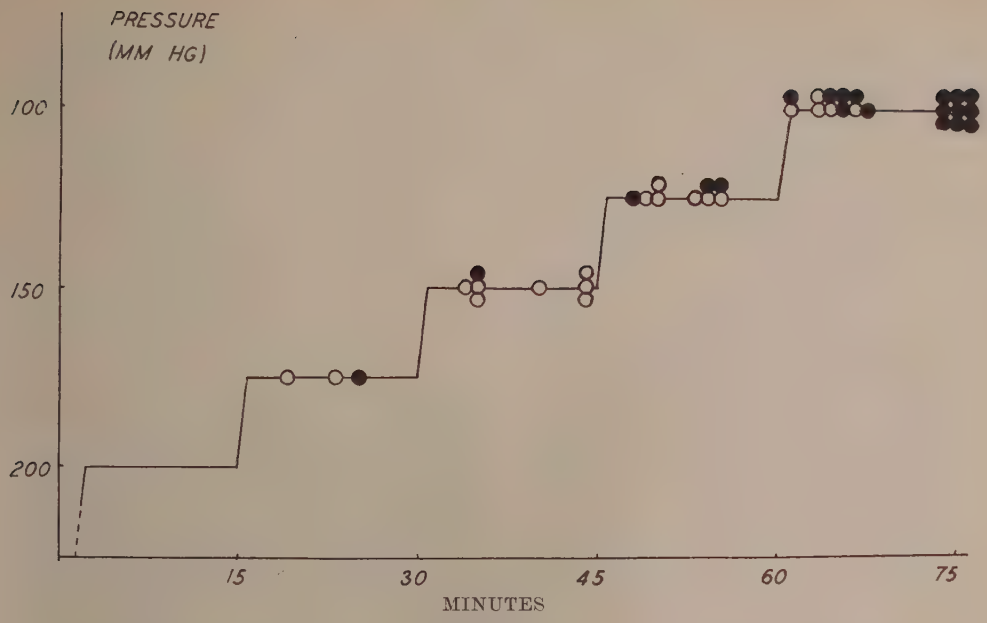

FIG. 1.

Survival of normal and thyroidectomized rats at various levels of reduced pressure. Each circle indicates the death of a rat; the light circles referring to the controls and the dark circles to the operated animals.

fasted animals, whether normal or thyroidectomized, were considerably less resistant to anoxia than fed animals, no differences between intact and operated animals being apparent in the fasted groups. However, the fed thyroidectomized rats withstood anoxia much better than the fed controls.

In all the following experiments (Table II), the animals were fasted overnight, fed during one hour, and exposed to the low pressures 3 to 4 hours later. The presence of a large amount of food in the stomach was ascertained at autopsy. The greater resistance to anoxia in thyroidectomized animals was confirmed, using a group of 20 rats operated from 6 to 8 weeks previous to the test (Table II, Fig. 1). Another group of rats received a 1% solution of thiourea in drinking water for 3 months; these proved much more resistant to anoxia than their controls (Table II). No controls but almost half the thyroidectomized and thiourea-treated rats survived to the end of the test. On the other hand, the resistance to oxygen deficiency was markedly decreased in rats treated either by 0.2 mg of thyroxine daily for 3 weeks or by a single injection of 25 mg of dinitrophenol per 100 g of body weight 30 minutes before exposure to the low oxygen pressures (Table II).

The opposite effects of thyroidectomy and thyroxine treatment on resistance to anoxia were probably mediated through metabolic variations, since dinitrophenol, a metabolic stimulator without effect on thyroid defi-

TABLE I.

Resistance of Fed and Fasted Thyroidectomized Rats to Anoxia.

Duration of fast (hr)	Operation	Avg body wt (g)	Avg survival (min) ± standard error
3	None	125	55.5 ± 3.5
22	"	121	28.3 ± 4.2
3	Thyroidectomy	83	69.2 ± 3.9
22	"	79	31.4 ± 5.3

TABLE II.
Effects of Various Metabolic Agents on Resistance of Rats to Anoxia.

	No. of animals	Avg body wt (g)	Avg survival (min) ± standard error
Controls	20	192	47.3 ± 3.4
Thyroidectomized	20	118	64.0 ± 3.2
Controls	7	279	50.4 ± 2.7
Thiourea	9	155	68.0 ± 3.2
Controls	6	267	44.3 ± 3.2
Thyroxine	6	225	26.5 ± 4.1
Controls	6	193	44.2 ± 3.7
Dinitrophenol	6	197	12.1 ± 4.8

ciency,[15,16] decreases the resistance to low oxygen pressures as does thyroxine. However, in the animals fasted for one day, the effect of metabolic variations on anoxia resistance was overshadowed by the increased sensitivity to anoxia resulting from the fast.[8,9]

Conclusions. (1) Thyroidectomy increases the resistance of the rat to anoxia; but only in fed animals. (2) Treatment with thyroxine decreases the resistance of the rat to anoxia. Since dinitrophenol, a metabolic stimulator without effect on thyroid deficiency, also decreases the resistance to a low oxygen pressure, the role of the thyroid in conditions of anoxia appears mediated through the effect of this gland on metabolism. (3) Animals given a 1% solution of thiourea in drinking water show a marked increase in resistance to anoxia probably as a consequence of the suppression of thyroid function due to this drug.

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16 Leblond, C. P., in press.

14483

Inhibition of Growth of Typhus Rickettsiae in the Yolk Sac by Penicillin.

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The results to be reported here were obtained in one of a series of experiments designed to test the effects of various enzyme inhibitors and activators on the intracellular multiplication of rickettsiæ. The yolk sac of the developing hen's egg (Cox[1]) was chosen as the medium for this work because of its uniformity and economy.

Material and Methods. A murine strain of typhus rickettsiæ, in its 8th and 9th passages in fertile eggs, was used. 36 eggs were injected, 18 of which were treated with penicillin, while 18 served as controls. All eggs were incubated at 37°C.

A yellow powder containing the sodium salt of penicillin was dissolved in distilled water. In the first experiment 0.2 cc of this solution, containing 325 Oxford units, was injected into the yolk sacs of 6 eggs on the 2nd, 4th, and 6th days after injection with rickettsiæ. In the second experiment, similar injections were given to 12 eggs on the 3rd, 5th, and 7th days after injection with rickettsiæ.

The eggs were studied for the presence of rickettsiæ at intervals after injection. When an egg in either the control or the treated series showed evidence of fetal death, it was opened for study, and usually an egg in the corresponding series was opened and studied simultaneously. In Experiment 1, when the

1 Cox, H. R., *Science*, 1941, **94**, 399.

TABLE I.
Growth of *Typhus rickettsiæ* in Penicillin-Treated Eggs.
Experiment 1.

Control series				Penicillin series		
Day after inoculation	Egg No.	Degree of infection*	Embryo at time of examination	Egg No.	Degree of infection*	Embryo at time of examination
5				1	(+)?	dead
8	1	++	dead			
9	2	++	alive	2	(+)	alive
10	3	+++	”			
11	4	++++	dead			
	5	+++++	”			
12	6	++++	”	3	(+)	”
13				4	(+)	”
16				5	(+)	”
17				6	(+)	dead

* (+)? No *rickettsiæ* recognizable with certainty.
(+) Less than one *rickettsia* per oil immersion field.
\+ 1-10 *rickettsiæ* per oil immersion field.
++ 10-100 ” ” ” ” ”
+++ 100-1000 ” ” ” ” ”
++++ 1000-5000 ” ” ” ” ”
+++++ 5000-8000 ” ” ” ” ”

embryos of the last 3 control eggs died with overwhelming rickettsial infection, the remaining treated eggs were opened subsequently at varying intervals. Under the conditions of the experiment, eggs rarely hatched, even when not given injections of any sort.

The degree of infection was estimated in giemsa-stained smears from the yolk sac membrane of each egg, and occasionally in giemsa-stained paraffin sections. The number of rickettsiæ was determined, in lightly infected eggs, by actually counting the organisms in a number of oil immersion fields. In heavily infected eggs, the rickettsiæ in a small portion of each of several fields were counted, and on this basis an estimate was made of the total number in each field. Although several sources of error are inherent in this method, it gave results sufficiently accurate for the purposes of this study.

On two occasions, material from one control and one treated egg was injected intraperitoneally into guinea pigs, in order to test for virulence. The yolk from one egg showing fetal death 9 days after the last injection of the drug was tested for penicillin activity against *Staphylococcus aureus*.

In order to rule out the possibility that the observed effect might be due to the injected distilled water, a supplementary series of 12 eggs was injected with rickettsiæ, and 6 of these were injected with 0.5 cc of distilled water 5 days later.

Results. From Tables I and II, it will be seen that the majority of the control eggs died with heavy rickettsial infection between the 9th and 13th days after injection. Of the 18 eggs in this series, one died on the 4th day—too early to be significant. In 3 eggs, the estimated number of rickettsiæ per oil immersion field was 10-100; in 2 eggs, 100-1000; in 5 eggs, 1000-5000; and in 7 eggs 5000-8000.

In the penicillin treated series, one fetal death occurred on the 3rd day, one on the 5th day, and 2 on the 6th day; all before the usual period for massive rickettsial growth. (Traumatic injury from successive injections may have caused some of these early fetal deaths.) In 9 eggs, the estimated number of rickettsiæ per field was less than one; in 3 eggs, 1-10; in one egg 100-1000; and in one egg 1000-5000.

Guinea pigs injected with material from 2 control eggs (heavily infected) and 2 treated eggs (containing less than one organism per field) all developed typical murine typhus infection. Penicillin activity could not be demonstrated in the yolk tested 9 days after the last injection of the drug.

Four of the 6 eggs in the supplementary series given injections of pure distilled water all showed massive rickettsial infection, quan-

TABLE II.
Growth of *Typhus rickettsiæ* in Penicillin-Treated Eggs.
Experiment 2.

Control series				Penicillin series		
Day after inoculation	Egg No.	Degree of infection*	Embryo at time of examination	Egg No.	Degree of infection*	Embryo at time of examination
3				1	(+)?	dead
4	1	(+)?	dead			
6				2	(+)?	"
				3	(+)?	"
8	2	+++	alive	4	(+)?	"
9	3	++	"	5	(+)	alive
10	4	++++	dead	6	(+)	"
11	5	++++ –	alive	7	(+)	"
12	6	+++++	dead	8	+	"
	7	+++++	"	9	++++	"
	8	+++++	"			
	9	+++++	"			
	10	+++++	"			
13	11	+++++	"	10	+++	dead
14	12	++++	alive	11	+	alive
15				12	+	"

* For explanation of symbols, see Table I.

titatively comparable to that seen in the control eggs. The other two died early with bacterial contamination.

Discussion. The positive inoculation test in the case of two eggs in which the number of rickettsiæ in smears was less than one per field, indicates that some of these organisms were alive. It is not clear whether these scattered rickettsiæ indicate a slight multiplication of the organisms, or merely represent the organisms originally introduced.

The eventual development of massive rickettsial infection in 2 of the treated eggs, 5 and 6 days after the last injection of penicillin, is not surprising in view of the evidence, obtained by guinea pig injection, that live organisms were present. The failure of this massive growth to occur with regularity after the cessation of treatment is probably to be explained by the fact that in the later stages of embryonic development, conditions in the entodermal cells lining the yolk sac become less favorable for rickettsial growth. It is probable that the penicillin, as given in these experiments, inhibited rickettsial growth during the critical period, so that the eventual development of massive infection was usually not possible.

It should be emphasized that penicillin, in all probability, is destroyed rather rapidly in the yolk sac, and that no attempt was made to maintain effective concentrations. The drug was given in 3 single doses rather than in many divided doses in order to lessen the danger of traumatic fetal death.

Although these experiments show a striking inhibition of rickettsial growth by penicillin, there is no evidence that rickettsiæ completely disappeared in any instance. The mechanism of the inhibitory effect on rickettsial growth is not clear at present. In general, rickettsial and viral infections are unaffected by chemotherapeutic agents of the sulfonamide group. The only exceptions of which we are aware are lymphogranuloma venereum,[2] mouse pneumonitis,[2] and trachoma.[3] In experimental typhus and spotted fever, negative or even suggestively detrimental effects from the sulfonamides have been reported.[4,5] Preliminary experiments in this laboratory indicate that sulfanilamide does not inhibit rickettsial growth in the yolk sac. Penicillin is reported to be ineffective against the influenza virus, PR8.[6]

Because of the fact that rickettsiæ and

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[4] Topping, N. H., *Pub. Health Rep.*, 1939, **54**, 1143.

[5] Pinkerton, H., *Bact. Rev.*, 1942, **6** (No. 1), 37.

[6] Robinson, H. J., *J. Pharm. and Exp. Therap.*, 1943, **77**, 70.

viruses are obligate intracellular parasites, studies of the effect of chemotherapeutic agents on these pathogens are complicated by several factors, which need not be considered in the case of ordinary bacteria. Unknown factors are (1) the permeability of living host cells to the agents, (2) their stability within cells, and (3) their effect on the enzyme systems of the cells. Since rickettsiæ and viruses are dependent in variable degrees upon the enzyme systems of the cells in which they grow, any observed effects on the growth of intracellular parasites might be either direct (comparable to the bacteriostatic effect upon free-living bacteria) or indirect, secondary to alterations of the metabolism of the host cells. It cannot be assumed, therefore, that the inhibitory effect of penicillin on rickettsial growth in the yolk sac is brought about necessarily in the way similar to the bacteriostatic effect of this substance on free-living bacteria.

Multiplication of rickettsiæ in the yolk sac takes place only with cells.[7] Penicillin was not injected until 48 or 72 hours after the injection of rickettsiæ, in order to allow time for the injected rickettsiæ to gain entrance into the cells. Careful attention was given to the cells in the yolk sacs of the penicillin-injected eggs, and with the exception of the 2 heavily infected eggs (Table II), only a rare isolated organism was seen in such a position that it might have been thought to be intracellular. The observations made strongly suggest that the inhibition of rickettsial growth is brought about by penetration of the penicillin into the cells, and not entirely by its direct effect on extracellular organisms in the process of passing from cell to cell.

The effect of penicillin injection on experimental typhus and spotted fever infection in mice and guinea pigs is being investigated and will be reported later. The experiments reported here suggest the possibility that penicillin might be effective in these diseases.

Conclusion. Penicillin, injected in 3 doses at intervals of 48 hours, exerted a striking inhibitory action on the multiplication of murine typhus rickettsiæ in the yolk sac of the fertile hen's egg.

For the penicillin used in these experiments, the authors are indebted to the National Research Council Committee on Chemotherapeutics and Other Agents.

7 Greiff, D., and Pinkerton, H., to be published.

14484

Glucose Tolerance of Fasted and Insulinized Chicks.

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The blood sugar level of fasted chicks exhibits a definite upward trend after 24 hours of fast, while 24 hours after large doses of insulin the blood sugar level is elevated above the fasting level.[1] The elevation of the blood sugar level after large doses of insulin is apparent after a single injection and becomes more marked after subsequent injections in either fasted or non-fasted chicks. The insulin sensitivity of such chicks may be tested by administering a small dose of insulin and observing the fall of blood sugar 1½ hours after injection, since the response is a logarithmic function of the dose up to 1.5 units per kilogram. The behavior of the blood sugar curve after large doses of insulin raised the question whether or not such chicks were less sensitive to small amounts of insulin. It has been shown in a previous publication that neither fasting nor dehydration, separately or together, would change the insulin sensitivity.[2] Such a desensitizing effect has been found, and a study of the glucose tolerance on fasted chicks receiving insulin and

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2 Opdyke, D. F., *Am. J. Physiol.*, 1943, **139**, 563.

TABLE I.

A comparison of the insulin sensitivity of chicks fasted 72 hours with that of chicks fasted 72 hours and, in addition, receiving 3 large doses of insulin during the fast. The insulin was administered in 20-unit doses at the beginning and after 24 and 48 hours of fast. Insulin sensitivity was determined by injecting 0.18 unit of insulin and measuring the blood sugar 1½ hours afterward.

Experimental condition	No. of chicks	Blood glucose, mg %		
		Control blood sugar	90 min. after injection of test dose	% change in blood sugar
Fasted 72 hrs. 0.18 u. insulin test dose	29	181 ± 3.5*	140 ± 3.6*	—22.6 ± 1.9*
Fasted 72 hrs. 3 inj. of 20 u. insulin day. 0.18 u. insulin test dose	28	207 ± 10.0	180 ± 12.0	—13.0 ± 3.5

* Mean and standard error.

without insulin was instituted.

Procedure. The chicks used in this study were cockerels of the single comb White Leghorn variety. They were obtained from the hatchery when one day old and were raised on a diet of Wayne or Arcady chick mash to an age of 30-40 days. Their body weight ranged between 200 and 400 g, with an average weight of 260 g.

Blood sugars were determined by the method of Hagedorn and Jensen as given by Peters and Van Slyke.[3] The blood was obtained directly from the wing vein. The chicks were individually marked so that the time between samples could be very accurately controlled.

Glucose (2 g/kilo, 10% solution) was administered by introducing the free end of a rubber tube attached to a graduated burette well down into the crop. The amount of glucose solution given was adjusted to the nearest 10 g of body weight. No regurgitation occurred if the tube was carefully withdrawn. A control blood sugar sample was taken immediately before the glucose solution was given. Blood samples were also taken 20, 40, and 80 minutes after the administration of glucose.

Insulin* was injected into the pectoral muscle. The test dose was 0.18 unit in a volume of 0.5 cc of fluid. Dilutions were made with acidified distilled water, pH 2.4.

Results. Tables I and II summarize the results of this study.

Table I clearly indicates that the previous injection of large doses of insulin decreases the sensitivity of chicks to a small dose of the same hormone. The fasted and insulinized chicks exhibit the typical increase of the blood sugar level above the fasting level, and the percentage change in blood sugar following the test dose of insulin is significantly less than that found in the chicks which were only fasted without receiving any insulin previously.

If the data presented in Table II are studied graphically, striking differences in the character of the glucose tolerance curves become obvious. The blood sugar of the non-fasted series has returned to its original level 80 minutes after the ingestion of glucose, the highest level being obtained after 20 minutes. After 24 hours of fast the blood sugar fails to return to its original level at the end of 80 minutes. The difference between the original value and that at 80 minutes is highly significant. In addition, the level of the blood sugar 20 minutes after the administration of glucose is significantly higher in the 24-hour fasted series than in the non-fasted series. The 72-hour fasted group presents a peculiar feature in that the curve is greatly flattened. The blood sugar level fails to reach the level found in the 24-hour fasted series, although it is still above the initial level at the end of the 80-minute period.

The most marked change in the character of the response occurs after administration of large doses of insulin. Here, 24 hours after a single injection of 20 units of insulin, the control blood sugar is elevated; the peak occurs 40 minutes after the administration of

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* The insulin (Iletin) was kindly furnished by Eli Lilly and Co., Indianapolis, Indiana.

TABLE II.

Glucose tolerance of non-fasted, fasted, and insulin-treated fasted chicks. All chicks received 2 g glucose per kilo. The insulinized chicks received one injection of 20 units of insulin per day of fast. The glucose tolerance of the insulin-treated chicks was determined 24 hours after the last insulin injection.

Experimental conditions	No. of chicks	Hours fasted	Blood glucose in mg % after 2 g glucose/kilo: Control before glucose	20 min	40 min	80 min
Non-fasted	10	0	201 ± 3.1*	241 ± 8.1*	211 ± 3.9*	199 ± 4.3*
Fasted only	15	24	179 ± 3.5	274 ± 10.0	220 ± 5.7	193 ± 3.3
" "	15	72	178 ± 2.9	227 ± 7.3	226 ± 8.3	197 ± 4.3
Fasted and receiving 20 units insulin 24 hrs prior to test	5	24	194 ± 27.0	239 ± 14.0	270 ± 22.0	255 ± 34.0
Fasted and receiving inj. of 20 u. insulin at 0, 24, and 48 hrs of fast	15	72	224 ± 13.0	313 ± 14.0	338 ± 13.0	344 ± 6.4

* Mean and standard error.

glucose, and the blood sugar remains markedly elevated at the end of 80 minutes. With 72 hours of fast and three large injections of insulin the shape of the curve is changed even more radically. In the latter case, the blood sugar level is still rising at the end of the 80-minute period. Twenty minutes after the administration of glucose the blood sugar has reached a concentration not attained at any time in the preceding experiments, *i.e.*, 313 mg % as compared with 270 mg %. It is evident that the previous injection of insulin exerts a powerful influence on the character of the glucose tolerance curve.

Discussion. The results of the glucose tolerance tests on fasted chicks suggest the phenomenon of hunger diabetes which is well known in dogs, human beings, and rabbits.[4-6] The theory has been advanced that hunger diabetes results from the failure of the islet tissue of the pancreas to secrete insulin in the absence of ingested carbohydrate. Such a theory is supported by the experiments of Best *et al.*[7] which show that the insulin content of the pancreas of fasted or fat-fed rats is less than that of normally fed rats. A failure of the blood sugar of the fasted chick to return to normal after glucose ingestion might be explained on this basis. However, such an interpretation neither explains the marked flattening of the glucose tolerance curve after 72 hours fast, nor does it explain why the level of the blood sugar fails to reach the height attained after comparable treatment at the end of a 24-hour fast.

The very marked decrease in tolerance to glucose exhibited by chicks previously injected with large doses of insulin indicates a major change in the operating economy of these animals. Similar results have been reported on rabbits when glucose was given immediately after recovery from a hypoglycemia induced after injection of 1½-2 units of insulin.[6] Conceivably, such experimental conditions may upset the dynamic balance between the production of carbohydrate from endogenous sources and the utilization of that carbohydrate. Whether fasting decreases the utilization of carbohydrate is still a debatable question. The experiments of Bergman and Drury[8] on eviscerated fasted and fed rabbits indicates a lowered utilization rate of carbohydrate in the fasted animals. The progressively rising blood sugar level of the fasted chick and the augmentation of this rise by a single or repeated injection of insulin may be interpreted as an increase in the production

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of carbohydrate, a decrease in its utilization, or both. Possibly, both effects are present at different times, the overproduction phase representing a secondary reaction to the injection of insulin and manifesting itself as an increased blood sugar 24 hours after the last injection of insulin.

Summary. Normal chicks exhibit the usual type of glucose tolerance curve, but one shorter in duration when compared with the mammalian curves. The character of the curve begins to change after 24 hours of fast and is markedly changed when taken after 72 hours of fast. Previous injections of large doses of insulin greatly decreases the glucose tolerance.

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Influence of Atmospheric Temperature upon Reaction of Rabbits to Insulin.*

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The higher mortality among white rats and white mice when kept at atmospheric temperatures considerably higher than normal, after receiving injections of insulin, and their increased inclination to convulse has frequently been reported.[1-5] Others have found, however, that the inclination of white mice to convulse is the same at 30°C as at 18°-20°C.[6] It has also been found[7] that rabbits acclimated at a temperature of 30°C required but one-half as much insulin to produce the same percentage of blood sugar reduction as those at 22°C.

It was observed in the present experiments that rabbits which responded with mild convulsions, when injected with a prescribed amount of insulin at normal room temperatures, collapsed completely with the first onset of convulsions and expired forthwith after being injected with the same amount of insulin during extremely hot weather (31° to 34°), if not given immediate intravenous injections of glucose. The rabbits used were divided into 3 groups, A, B, and C. Those of group C were inclined to convulse after injections of 0.375 units of insulin per kg of body weight, those of group B with 0.5 units, while those of group A did not convulse with the larger amount. They were used but once a week and were fasted for 18 hours before each experiment. The dosage of insulin in each of these experiments was 0.375 units of crystalline zinc insulin† per kg of body weight.

Samples of blood were taken from each animal just before it was injected with insulin and similar samples taken each hour thereafter for 7 hours to determine the nature of the subsequent changes in the blood sugar level. Blood sugar determinations were made on individual samples according to the method of Miller and Van Slyke.[8] Group averages of the data thus obtained are represented by the graphs of Fig. 1.

* The author is greatly indebted to the donor of the Walter C. Hadley Fund whose support helped to make this investigation possible.

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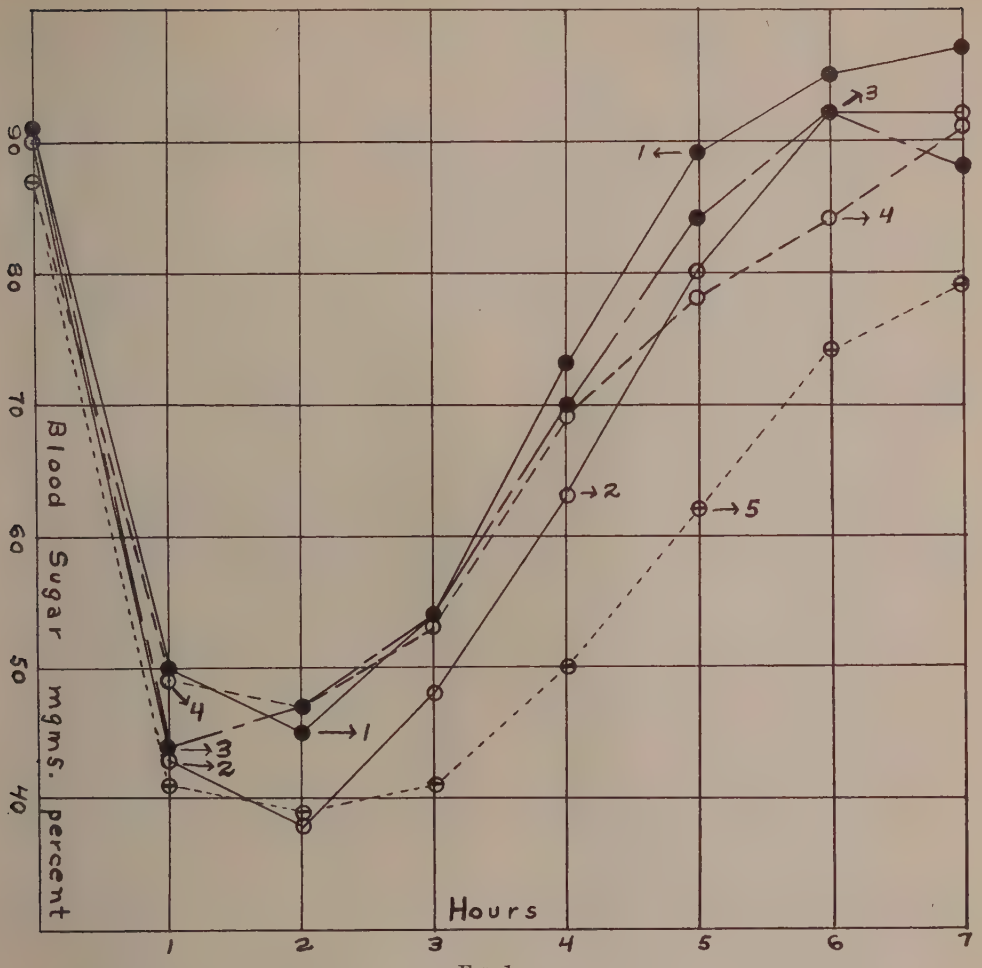

FIG. 1.

Curves 1-4 show the progressive changes in the blood sugar of rabbits not inclined to convulse after injections of insulin.

	Temp. of 18-hr fasting period	Temp. of 7-hr experimental period
Curve 1	33°C	33°C
" 2	20	20
" 3	33	20
" 4	20	33

Curve 5, obtained under similar conditions as curve 3 with rabbits less inclined to convulse under conditions pertaining to curves 1 and 2, shows a persistently lower level of blood sugar.

No animal of group A (8 rabbits) or of group B (3 rabbits) was ever inclined to convulse under any of the conditions of the present experiments. When the animals of group C (3 rabbits) were kept at 33°C or at 20°C during the 18-hour fasting period as well as during the subsequent 7-hour experimental period all were invariably inclined to convulse. When, however, these same animals were kept at 33°C during the 18-hour fasting period and at 20°C during the 7-hour experimental period it was found in repeated experiments with each animal that none of them convulsed. The blood sugar level reached

under these circumstances was fully as low as that reached under conditions when the animals convulsed. The data obtained with group C was averaged with similar data obtained with group B (graph 5).

When the animals of group A were kept at 33°C during both the 18-hour fasting period and the 7-hour experimental period it was found that their blood sugar level was consistently higher than when they were kept at a temperature of 20°C during these periods. (Graphs 1 and 2.) This may be due to a lack of complete acclimatization to the lower temperature. Recent data indicate that 3 to 4 days are required for the full acclimatization to desert temperatures by man.[9] Similar experiments with group B produced similar data. These data were not averaged with those obtained with group A because of occasional sporadic deviations in blood sugar values. This was also true of the data obtained with the rabbits of group C.

When the animals of group A were kept at 33°C during the 18-hour fasting period and at 20°C during the 7-hour experimental period the blood sugar level at first was inclined to follow the level maintained when these animals were kept at 20°C during both periods and then approached the level obtained when these animals were kept at 33°C during both periods. (Graph 3.) When the animals of group A were kept at 20°C during the 18-hour fasting period and at 33°C during the 7-hour experimental period a similar reversal of the blood sugar level, but in the opposite direction, took place. (Graph 4.) Similar data were obtained with the animals of group B.

The data which were obtained with group B when kept at 33°C during the 18-hour fasting period and at 20°C during the 7-hour experimental period were averaged with those obtained under similar conditions with the rabbits of group C. (Graph 5.)

Phenomena associated with the failure of the rabbits of group C to convulse when kept at 20°C during the 7-hour experimental period after they had been kept at 33°C during the 18-hour fasting period, are suggestive of similar phenomena associated with the behavior of parathyroidectomized animals under the influence of a sudden change in the acid-base balance of the blood. Because of the base deficit caused by overventilation at 33°C the rabbits would be subject to an uncompensated acidosis at 20°C until the kidneys had restored the acid-base balance to a normal state.

Just as an increased acidity restrains the onset of tetany in parathyroidectomized animals it appears to restrain the inclination of normal animals to convulse under the influence of insulin hypoglycemia. An inhibiting effect upon the convulsive response after insulin injections due to an atmosphere with a high partial pressure of CO_2 has been reported.[10]

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14486

Androgen Assay on Three-Day-Old Male Chicks.*

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The capon comb growth method is recognized as the standard procedure for the biologic assay of androgens. However, when a great many assays are being performed constantly the difficulties of maintaining large numbers of such birds diminishes the practicability of this method.

Frank and his associates have developed an assay method based on the comb growth of the immature chick. The progress in devel-

oping the method was described in a number of reports[1-4] with a final detailed description of the procedure and method of calculation.[5] Before this detailed report appeared a number of investigators had used the immature chick for assay purposes[6,7,8] but the results were unsatisfactory and the method was considered unreliable. This is believed by Frank *et al.*[9] to be due to failure to adhere strictly to the details of the procedure specified by them.

We were led by the reports of Frank *et al.* to use the immature chick for androgen assays; our procedure was developed on the basis of their earlier communications and therefore differs in certain respects from the method finally outlined by these authors.

Method. Single Comb White Leghorn chicks are purchased from one source. The chicks are shipped on the first day of life and arrive at the laboratory the following morning. The assays are started the next day (*i.e.*, when the chicks are from 48-72 hours old). Although a slight variation (less than 24 hours) in age is unavoidable, the influence of this factor is minimized by performing control assays with each batch. Only male chicks are used, obviating the necessity for statistical correction for differences in comb growth response of males and females. Repeated checks by autopsy have demonstrated the reliability of the selection of "sexed" cockerels by the hatchery (within 5%).

On arrival, the chicks are placed in a thermo-regulated brooder. Frank *et al.* emphasize the importance of constant lighting conditions but recent reports[10] indicate that it is the temperature rather than the light that is of importance in this connection. The cockerels are kept in a room with ample daylight, but since control groups are run with each batch, the results are not influenced by variations in this factor.

Vehicle for Hormone Solution. The androsterone‡ and the extracts to be assayed are dissolved in ether. The ether solution is applied directly to the lateral surface of the comb by means of a fine needle and microsyringe or tuberculin syringe fitted with a micrometer screw. The applications are best accomplished by having one worker hold the comb firmly while the other manipulates the syringe so that the ether solution does not spread beyond the comb before drying. Applications are made once daily for 7 days and the animals are killed on the following day, the 8th day of the experiment or the 10th day of life. For the past 2 years we have employed ether rather than oil as a vehicle for applying the hormones following a suggestion made by Koch[11] for the assay of small amounts of androgens on capons. Since the ether evaporates quickly, the danger of the solution spreading beyond the comb or being smeared from one animal to another is avoided. Frank *et al.* prevent smearing from one group to another by isolating each group, but this obviously does not prevent transfer from one animal to another within the same group. When performing large numbers of assays we have not been able to individualize each group because of lack of space, but control experiments have demonstrated that this is not

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11 Koch, F. C., read at 25th Annual Meeting of the Association for the Study of Internal Secretions, May 21, 1941, Atlantic City, New Jersey.

TABLE I.
Comb-Bodyweight Ratios of Chicks Treated with Androsterone.

Dosage of Androsterone: γ per cc	0	5	10	20	50	100	250
γ total dose	0	1.75	3.5	7.0	17.5	35.0	87.5
1.	26.9* ± 2.3† (8.2)‡	61.6 ± 16 (25.4)	—	76.3 ± 10 (13.1)	—	—	—
2.	27.4 ± 5.1 (18.5)	45.4 ± 7.7 (16.9)	54.9 ± 7.1 (12.9)	78.8 ± 10.4 (13.3)	—	—	—
3.	23.6 ± 3.4 (14.0)	36.3 ± 3.9 (11.0)	44.2 ± 4.0 (9.0)	76.6 ± 9.4 (12.2)	—	—	—
4.	23.7 ± 4.1 (17.2)	46.9 ± 11.7 (24.9)	64.0 ± 11.2 (17.5)	69.9 ± 10.9 (15.5)	—	—	—
5.	26.1 ± 6.3 (24.1)	53.5 ± 11.1 (20.7)	68.9 ± 15.6 (22.6)	—	—	—	—
6.	23.8 ± 3.6 (15.1)	44.5 ± 5.8 (13.0)	57.3 ± 8.4 (14.6)	67.4 ± 9.5 (14.0)	—	—	—
7.	22.1 ± 4.1 (18.5)	—	—	44.6 ± 7.7 (17.2)	59.9 ± 5.8 (9.6)	92.4 ± 18.4 (19.9)	123.8 ± 26.2 (21.1)
8.	25.2 ± 1.5 (5.9)	—	44.4 ± 5.9 (13.2)	56.0 ± 11.1 (19.8)	80.7 ± 15.8 (19.5)	—	—
9.	28.1 ± 6.3 (22.4)	36.9 ± 3.6 (9.7)	52.5 ± 8.4 (16.0)	64.9 ± 5.6 (8.6)	—	—	—
10.	23.4 ± 4.3 (18.3)	31.6 ± 5.9 (18.6)	39.5 ± 6.5 (16.4)	48.7 ± 9.8 (20.1)	68.1 ± 14.8 (21.7)	89.1 ± 11.2 (12.5)	—
11.	22.2 ± 1.9 (8.5)	41.1 ± 4.4 (10.9)	61.1 ± 14 (22.9)	78.6 ± 13.2 (16.8)	—	—	—
12.	27.2 ± 4.9 (18.0)	—	—	57.1 ± 12.0 (21.0)	81.5 ± 23.4 (28.7)	116.3 ± 25.4 (21.8)	159.7 ± 22.3 (13.9)
13.	20.5 ± 5.2 (25.3)	32.3 ± 9.8 (30.3)	45.1 ± 9.9 (21.9)	—	70.3 ± 13.5 (19.2)	—	—
14.	26.7 ± 5.7 (21.3)	48.2 ± 13.3 (27.5)	62.6 ± 12.3 (19.6)	82.9 ± 18.8 (22.2)	—	—	—

* Comb-body weight in mg per 100 g.

† Standard Deviation $\sigma = \sqrt{\frac{\Sigma (\bar{x})^2}{n-1}}$, $n = 10$

‡ Coefficient of variation.

necessary when ether is used as the vehicle.

Standardization. The androsterone controls are assayed on groups of 10 chicks each, at 3 or more dosage levels within the range of satisfactory response (Table I). The unknown is similarly assayed on groups of 10 chicks, whenever possible using more than one dilution, depending upon the amount of material available and the expected response. In addition, on one group of 10 cockerels, only the vehicle, ether, is used.

Results. In this report are presented the results of assays of the androsterone control solutions in 14 experiments involving 2800 chicks, 620 of which received various dosages of androsterone. The pertinent data are presented in Table I.

The average combweight in mg per 100 g bodyweight of the controls receiving the vehicle (ether) only, varied from 20.5 ± 5.2 to 27.0 ± 5.1 at the end of the experimental period. In 10 experiments in which each bird in one group received a total of 3.5 γ of androsterone, the average combweight per 100 g bodyweight varied from 39.0 ± 6.5 to 64.0 ± 11.2 mg. Variations of the same magnitude were observed at all dosage levels employed.

We do not know the cause of these variations in sensitivity of the comb response. However it is clear that the differences in average combweight response in the different experiments are the result of a true variation in sensitivity and are not due merely to a higher original combweight. If the latter were the case the groups showing the highest comb-body weight ratios among the ether controls would also show the highest values at the

various dosage levels. The slope of the curves would be the same in all experiments. However there is no such parallelism between comb-body weight ratios of controls and treated animals, and consequently the slopes of the curves vary. Therefore, corrections for initial bodyweight cannot correct for these variations. For these various reasons the use of a composite standard dose-response curve gives less accurate results than the construction of individual dose-response curves for each assay.

Dose Range. The total dosages used in the 14 experiments ranged from 0.175 γ to 87.5 γ. Not all dosage levels were employed in each experiment. A dosage of 1.75 γ (5 γ per cc) was used in 11, 3.5 γ (10 γ per cc) in 11, 7 γ (20 γ per cc) in 12 and 17.5 γ (50 γ per cc) in 5 experiments. The other dosage levels were employed in less than 5 experiments. Most of the curves are linear within the dosage levels employed, but some show a flattening at the higher dosage levels.

In a number of experiments not reported in this paper, in which testosterone and methyl testosterone were also employed, testosterone was found to give a response of about the same magnitude as androsterone, while methyl testosterone assayed somewhat higher.

Summary and Conclusions. Androgen assay by means of the comb growth response of the immature cockerel was found satisfactory. The method described is based upon that of Frank and his associates, but in addition to minor modifications, differs from the latter in the following respects: (1) male chicks only are used; (2) ether is employed as the vehicle for application of the hormone; (3) individual dose-response curves are constructed with each assay.

14487

Excretion in the Dog of Androgens and Estrogens in the Bile Following Injections of Androgens.*

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Testis transplants into the mesentery[1,2] and testosterone pellets implanted in the same situation[3] or in the spleen[4,5] fail to exert androgenic activity in castrate male rats, as evidenced by the lack of repair of the prostate and seminal vesicles. It has been concluded from such experiments that testosterone is inactivated by the liver.

We have shown in previous communications[6,7,8,9] that large amounts of estrogenic hormones both of exogenous and endogenous origin are excreted in the bile. Because of these observations it was decided to study the excretion of androgen and estrogen in the bile following injection of androgenic hormones in the dog. In this species administration of

* Aided by a grant from the Johnson & Johnson Research Foundation, New Brunswick, N.J.

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TABLE I.
Excretion of Androgens and Estrogens in Bile and Urine Following Injection of Androgens.

Exp. No.	Injected mg	Hr	Excreted in bile: Androgens* free	Androgens* total	Estrogens† free	Estrogens† total	Excreted in urine: Androgens free	Androgens total	Estrogens free	Estrogens total
1. Androsterone	50	24	3.2							
		48	1.6							
		72	0.0							
2.	50	24	7.2							
		48	4.0							
3.	80	24	0.25	0.2	0	0	.8	.6	6	20
		48	0.25	0.2	4	0§	.0	.0	0	0
		72	0.08		3	0	.0	.0	0	0
4.	10	24	0.15	0.11						
		48	0.0	0.0						
		72	0.0	0.0						
5. Testosterone	100	24	8.4							
		48	2.0							
		72	0.0							
6.	100	24		0.6		0		.9		40
		48		0.3		20		.2		40
		72						.075		0
7.	100	24	1.5	3.0		0	.8			40
		48	0.9	0.9		0	.6			40
8.	92	24		2.0				.0		
		48		1.6				.0		
9.	83	24	1.1	0.9	40	40	.0	.2	0	0
		48	0.4	0.3	0	4	.0	.1	4	0
		72	0.4		0	0	.0		0	0
10.	50‡	24	0.44	0.48	0	0	.0	.1	24	24
		48	0.0	0.1	20	13	.0	.0	13	13
		72			0	0	.0	.0	0	0
11.	50	24	2.65							
		48	0.75							
12.	10	24								
		48	0.19	0.19	6	6				
		72	0.0	0.0	0	0				
13. Methyl test.	100	24		3.8		0		.65		0
		48		1.5		0		.50		0
		72		1.8		0		.25		0
14.	100	24	0.6	0.6	0	0	.06	.0	100	0
		48	0.06	0.05	20	0	.0	.0	30	0
		72			0	24			0	0
15.	10	24	0.2	0.3	0					
		48	0.2	0.2	0					
		72	0.0	0.0	0					

* Androgens expressed as mg androsterone.
† Estrogen values given in I.U. of estrone.
‡ In this experiment testosterone was given in bile through duodenal fistula.
§ Values for free estrogens higher than those for total may be attributed to destruction in the process of hydrolysis.

androgens is not followed by increased excretion of androgens or 17 ketosteroids in the urine.[10,11] However, in the dog, as in humans, injection of androgens is followed by excretion of estrogens in the urine.[11,12]

Material and Methods. Fifteen experiments were performed in 9 dogs with external bile fistula, the bile draining into a balloon. The dogs were fed purina dog chow and meat, supplemented by cod liver oil and yeast extract. About 100 cc of bile was re-fed daily, either by stomach tube or through a duodenal fistula. During the experimental periods, the

[10] Kochakian, Ch. D., *Endocrinology*, 1937, **21**, 60.

[11] Paschkis, K. E., Cantarow, A., Rakoff, A. E., Hansen, L. P., and Walkling, A. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 213.

[12] Kochakian, Ch. D., *Endocrinology*, 1938, **23**, 463.

test animals received bile from untreated dogs, their own bile being removed for assay. Testosterone,‡ methyl testosterone,‡ and androsterone‡ in doses of 10 to 100 mg were injected intravenously in 2 to 3 cc 95% alcohol. In one experiment testosterone was dissolved in 2 cc ether, the ether was mixed with 100 cc bile, and the mixture was fed through a duodenal fistula in 3 divided doses.

For assay of free androgen, the bile collected over a 24-hour period, or an aliquot thereof, was extracted with ether and alcohol.

For total androgen the bile was hydrolized at pH 1 for 10 minutes either with sulfuric or with hydrochloric acid and subsequently extracted with butyl alcohol. After removal of the estrogens with sodium hydroxide the butyl alcohol was evaporated and the residue taken up in ether for assay. With this procedure 70 to 94% of added androsterone and testosterone was recovered from bile. Assays were performed on 3-day-old male chicks by a method described elsewhere.[13] Estrogens were assayed by Fluhmann's method as outlined in a previous paper.[7]

Results and Comment. The data are summarized in Table I. Androgenic substances were present in the bile following a single injection of testosterone, methyl testosterone, or androsterone. Control samples of bile exhibited no androgenic activity. Androgens were excreted in the majority of cases not only during the first 24 hours, but also during the second 24 hours following administration, and in a few experiments, also in the third 24-hour period, all of the bile being removed and only androgen-free bile re-fed. No increase of androgen excretion was demonstrable in the urine during the experimental period.[11] In preliminary experiments we have found that androgens are removed rapidly from the blood following a single intravenous injection, no androgen being demonstrable in the blood at the end of 24 hours. Consequently, androgen excreted in the bile during the second and third 24-hour periods must have its origin in androgen stored in some tissue.

All of the androgen was in the free state in 7 of 8 instances in which both free and conjugated androgens were assayed in the bile.

Androgens were excreted in the bile in every instance, the absolute amounts and percentage recovery varying considerably. The highest excretion occurred in Experiment 2, in which, following injection of 50 mg androsterone, 7.2 mg equivalents of androsterone was excreted in the bile during the first 24 hours and 4 mg during the second 24 hours. If the excreted androgen is androsterone, 22% of the injected material was recovered. On the other hand, a total of only 0.6 mg or 0.75% of the 80 mg injected was recovered in Experiment 3.

One experiment is included in the table in which testosterone was instilled into the duodenum through a duodenal fistula. Androgen was excreted in the bile, indicating that testosterone was resorbed from the intestine in the dog as it is in humans[14] and in capons.[15]

The figures for excretion of androgens probably represent only a fraction of the excreted compounds since part of the compounds excreted in the dog's bile may have little or no biological activity. Injection of testosterone propionate into humans is followed by excretion of androsterone and etiocholanolone in equal amounts in the urine.[16]

Comparison of the biliary excretion of androgens following androgen injection with that of estrogens following estrogen injection in the dog[6-9] reveals qualitative similarities but quantitative differences. Excretion of both androgens and estrogens continues for 2 to 3 days, during much of which time no hormone is found in the blood or in the urine. However, the quantity of androgen recovered

‡ Testosterone and methyl testosterone used in this study was generously supplied by the Schering Corporation through the courtesy of Dr. E. Schwenk and Dr. M. Gilbert, androsterone by the Ciba Corporation through the courtesy of Mr. R. Mautner.

[13] Rakoff, A. E., Paschkis, K. E., and Cantarow, A., submitted for publication.

[14] Dorfman, R. J., and Hamilton, J. B., *J. Clin. Invest.*, 1939, **18**, 67.

[15] Emmens, C. W., and Parkes, A. S., *J. Endocrinology*, 1939, **1**, 323.

[16] Callow, N. H., *Biochem. J.*, 1939, **33**, 559.

from the bile is much smaller in terms of bioactivity than that of estrogen.

Excretion of Estrogen in Bile Following Androgen Injection. Normal dog bile exhibits no estrogenic activity.[6] In 6 of 9 experiments estrogenic activity was demonstrated in the bile following injection of androgens. In 5 of these, the bile was examined for both free and conjugated estrogen, all being found to exist in the free state. We have demonstrated estrogens in the urine of dogs following androgen administration,[11] contrary to earlier reports.[12] It is noteworthy that estrogens appear in the urine during the first 24 hours following androgen administration, whereas, except in one case, they first appeared in the bile *after* 24 hours.

The source of estrogen in the bile and urine after administration of androgen is not known. It does not seem to be produced in the testes or adrenals.[11] The bile fistula dogs employed in the present study were females and the ovaries might have been stimulated by the injected androgen to secrete estrogen. However, the fact that estrogen was present in the urine following injection of testosterone in normal and castrate male dogs militates against this assumption. A portion of the injected androgen may have been converted into an estrogenic compound.[17,18] The total amount of excreted estrogen (bile and urine) was 100 i.u. in Experiment 6 (following the administration of 100 mg testosterone), 57 i.u. in Experiment 10 (following the administration of 50 mg testosterone), and 150 i.u. in Experiment 14 (following injection of 100 mg methyl testosterone). If all of the estrogen was estrone this represents transformation of 0.01 to 0.15% of the administered androgen. Hamilton and Dorfman[17] calculate the recovery from urine in humans as 0.04% of the administered androgen. In view of the probable presence of estrogen in the bile of these subjects, not included in this calculation, it would appear that production of estrogen following administration of androgen (conversion of androgens to estrogen?) is greater in humans than in dogs.

Summary and Conclusions. 1. In bile fistula dogs a single injection of androsterone, testosterone, or methyl testosterone is followed by excretion of androgenic material in the bile. Excretion of small amounts of androgenic material may continue for 72 hours in spite of the fact that all of the androgen-containing bile is removed from the body. 2. The quantities excreted vary considerably in different experiments with either of the 3 compounds studied. 3. A single injection of androgen is frequently followed by excretion of estrogen in the bile. 4. In one instance androgen and estrogen were excreted in the bile following intraduodenal instillation of testosterone.

[17] Hamilton, J. B., Dorfman, R. J., and Hubert, G. R., *J. Lab. Clin. Med.*, 1942, **27**, 917.

[18] Hoskins, W. H., Coffman, J. R., Koch, F. C., and Kenyon, A. T., *Endocrinology*, 1939, **24**, 702.

14488

Effect of Ascorbic Acid on Chick Growth when Added to Purified Rations.*

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It has long been recognized that chicks do not develop symptoms of scurvy when fed rations low or devoid of ascorbic acid.[1-8] For example, Hart, Steenbock, Lepkovsky, and Halpin[7] reported that chicks fed either a natural or a semi-purified ration did not develop scurvy and had an abundant supply of ascorbic acid in their livers. Carrick and Hauge[6] also found that livers of chicks on a scorbutic diet were high in ascorbic acid. In spite of this work the presence of a "scurvy-like" disease in chicks which was prevented by the inclusion of dried skim milk in the ration and cured by the addition of cabbage

was reported by Holst and Halbrook.[9] This work could not be confirmed by Cribbett and Correll[10] and in the light of recent investigations it is clear that Holst and Halbrook were probably dealing with a deficiency of vitamin K instead of ascorbic acid. Bell, Satterfield, and Cook[11] reported recently that a leg paralysis and other symptoms of a deficiency in 4 hens "under the demands of heavy egg production and fed a diet deficient in ascorbic acid" were cured by subcutaneous injections of ascorbic acid. In this paper we report the growth-promoting activity of ascorbic acid when added to certain *highly purified* chick rations.

Day-old White Leghorn chicks were placed in electrically heated cages with raised screen bottoms, fed the basal ration for 2 to 3 days, and then divided as uniformly as possible into groups of 6. The basal ration, 486K, as previously described in detail,[12] consists essentially of purified ingredients such as dextrin, casein, gelatin, soybean oil, salts, cystine, and known vitamins except ascorbic acid. (This ration has been modified to contain 20 γ of biotin per 100 g instead of 15 γ.) When ascorbic acid was used it was mixed in the ration in the powdered form unless otherwise noted. Water and the experimental ration, which was made up fresh weekly, were supplied *ad libitum*. This ration, 486K, is lacking in at least 2 vitamins, vitamin B_{10} and vitamin B_{11}, and possibly such factors as folic acid[13] (vitamin B_c[14]) which are supplied by solubilized liver when necessary. Without the solubilized liver chicks grow slowly, feather poorly, and become anemic. *p*-Aminobenzoic acid has been found to stimulate growth and feathering when added at high levels to this basal ration, although evidence has been presented that this compound is not a specific growth factor for the chick but acts indirectly by stimulating the production of unknown vitamins through intestinal synthesis.[12]

In Table I the results are given for the addition of ascorbic acid to the basal ration without solubilized liver and modified with various levels of *p*-aminobenzoic acid and other substances. Since the experiments were not performed at the same time, the results for each series of chicks are summarized in terms of "% of control weight," the control weight being that obtained with ration 486K plus 2% of solubilized liver. Neither ascorbic acid nor low levels of *p*-aminobenzoic acid, when fed alone to chicks on the basal ration, promoted growth, but when they were fed together at high levels the growth obtained

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We are indebted to Merck and Company, Rahway, New Jersey, for the crystalline vitamins including the ascorbic acid; to Wilson Laboratories, Chicago, Ill., for solubilized liver (liver fraction L) and whole liver substance; to Wilson and Company, Inc., Chicago, Ill., for gelatin; to the S.M.A. Corporation, Chagrin Falls, Ohio, for biotin concentrate No. 1000; and to Sharp and Dohme, Inc., Glenolden, Penn., for sulfasuxidine (succinylsulfathiazole).

[1] Hart, E. B., Halpin, J. G., and Steenbock, H., *J. Biol. Chem.*, 1922, **52**, 379.

[2] Plimmer, R. H. A., Rosedale, J. L., and Raymond, W. H., *Biochem. J.*, 1923, **17**, 787.

[3] Mitchell, H. H., Kendall, F. E., and Card, L. E., *Poultry Sci.*, 1923, **2**, 117.

[4] Emmett, A. D., and Peacock, G., *J. Biol. Chem.*, 1923, **56**, 679.

[5] Knox, C. W., and Lamb, A. R., *Poultry Sci.*, 1924, **3**, 101.

[6] Carrick, C. W., and Hauge, S. M., *J. Biol. Chem.*, 1925, **63**, 115.

[7] Hart, E. B., Steenbock, H., Lepkovsky, S., and Halpin, J. G., *J. Biol. Chem.*, 1925, **66**, 813.

[8] Hauge, S. M., and Carrick, C. W., *Poultry Sci.*, 1926, **5**, 166.

[9] Holst, W. F., and Halbrook, E. R., *Science*, 1933, **77**, 354.

[10] Cribbett, R., and Correll, J. T., *Science*, 1934, **79**, 40.

[11] Bell, T. A., Satterfield, G. H., and Cook, F. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 473.

[12] Briggs, G. M., Jr., Luckey, T. D., Mills, R. C., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 7.

[13] Briggs, G. M., Jr., Luckey, T. D., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1943, **148**, 163.

[14] O'Dell, B. L., and Hogan, A. G., *J. Biol. Chem.*, 1943, **149**, 323.

TABLE I.
Results of the Addition of Ascorbic Acid to the Basal Ration without Solubilized Liver Modified with Various Levels of *p*-Aminobenzoic Acid and Other Substances.

Supplement to ration 486K (per 100 g)	Avg wt of 6 chicks at 4 weeks				Avg % of control weight
	Series 1	Series 2	Series 3	Series 4	
	g	g	g	g	
No supplement (basal only)	140	136	142	127	61
2 g solubilized liver (control)	242	234	214	212	100
100 mg ascorbic acid	150	126			58
20γ *p*-aminobenzoic acid (P.A.B.A.)				134	63
50γ P.A.B.A.		146			62
100γ "		144		164	70
100γ P.A.B.A. + 100 mg ascorbic acid			180		84
500γ "		143	124		60
500γ P.A.B.A. + 20 mg ascorbic acid			196		92
500γ " + 100 " " "		196	151		78
1 mg P.A.B.A. + 100 mg ascorbic acid			164		77
10 " " + 100 " " "			204		95
500 mg sulfasuxidine		83		85	38
500 " " + 100 mg ascorbic acid		104			44
500 mg sulfasuxidine + 100γ P.A.B.A.				101	48
500 " " + 10 mg "				151	71
1 g whole liver substance			217		101
1 " " " " + 100 mg ascorbic acid			253	247	117
10 g whole liver substance			285		133

was nearly equal to that on solubilized liver. Only traces of these compounds are present in liver fractions which provide for normal growth of chicks on this ration.[13] Ascorbic acid counteracted the depressing effect of sulfasuxidine somewhat but *p*-aminobenzoic acid at high levels was most effective. Likewise, ascorbic acid stimulated growth when fed with a low level of whole liver substance, however, whole liver substance at a higher level, without added ascorbic acid, caused chicks to grow at a still higher rate than those on the control ration indicating the absence of unknown factor(s) even in the control ration.

The effect of ascorbic acid upon growth of chicks receiving the control ration was also determined (see Table II). Unexpectedly, it was found that the feeding of ascorbic acid gave a definite and consistent, although small, increase in growth when fed at 100 mg per 100 g of ration. Although administration of ascorbic acid by dropper and by injection as the sodium salt was partly effective, adding it to the ration in solution caused all of its activity to be lost, probably due to destruction in the ration itself.

Several other related substances were fed which, in a few instances, likewise caused growth. Arabinose, when fed at a level of 1%, promoted growth to the same degree as ascorbic acid. Other levels of arabinose have not been tested as yet. It is interesting in this connection that Stokstad *et al.*[15] have shown that certain carbohydrates, especially arabinose and glucuronic acid, were active in promoting growth of chicks receiving a semi-purified ration low in the carbohydrate portion of the "rice factor." It would be interesting to know if ascorbic acid could effectively replace the arabinose and glucuronic acid under the conditions of their experiments.

The growth rate of chicks receiving the control ration was stimulated also by the addition of 500 γ of *p*-aminobenzoic acid per 100 g of ration. When ascorbic acid was fed with *p*-aminobenzoic acid no additional growth was obtained over that observed with either one alone. This may indicate that both of these compounds are acting in the same manner. Sulfasuxidine, as reported previously,[12] caused no depression of growth when fed with the liver. In fact this drug for some reason, appeared to stimulate growth when fed alone

[15] Stokstad, E. L. R., Almquist, H. J., Mecchi, E., Manning, P. D. V., and Rogers, R. E., *J. Biol. Chem.*, 1941, **137**, 373.

TABLE II.
Results of Addition of Ascorbic Acid and Other Substances to the Basal Ration with Solubilized Liver.

Supplement to ration 486K with 2% solubilized liver (per 100 g)	Avg wt of 6 chicks at 4 weeks				Avg % of control weight
	Series 5	Series 6	Series 7	Series 8	
	g	g	g	g	
None (control)	242	248	234	191	100
1 mg ascorbic acid		250			101
50 mg ascorbic acid		251			101
100 " " "	318	281	263	224	118
500 mg ascorbic acid			243		104
1000 " " "			254		109
10 mg ascorbic acid per day by dropper			253		108
5 " " " " " inj. subc.			252		108
100 mg ascorbic acid (in sol. on ration)			233		100
100 " iso-ascorbic acid				194	102
1000 mg glucose				203	106
1000 " arabinose				223	117
500γ *p*-aminobenzoic acid	294		261		**117**
500γ " " + 100 mg ascorbic acid			257		110
500 mg sulfasuxidine			264		113
500 " " + 100 mg ascorbic acid			275		118
Practical ration (Wis. 45W)				196	103
" " + 100 mg ascorbic acid				202	106

while ascorbic acid when fed in addition to the sulfasuxidine was of little value in promoting further growth. Growth of chicks on a practical farm ration, low in or devoid of ascorbic acid, was not helped by the addition of this vitamin. This was not surprising when we consider that the natural products in the practical ration contain arabinose and certain other carbohydrates, which may have the same effect on growth as the ascorbic acid. Thus, the addition of ascorbic acid itself to the practical ration would be ineffective and unnecessary.

Preliminary investigations were made on the ascorbic acid content of the livers.† In 13-day-old chicks which had received 100 mg of ascorbic acid per 100 g of ration there were 35.9 mg per 100 g fresh liver and 20.0 mg in chicks that had received the control ration only. Livers of chicks at 4 weeks of age showed less difference, averaging 19.6 mg per 100 g fresh weight for the group receiving the ascorbic acid and 15.3 mg for the control group. This suggests either that excess ascorbic acid is being stored in the liver or, since growth was improved, that the rate of synthesis may not be fast enough in our chicks.

Discussion. It is difficult to determine from these data just how ascorbic acid stimulates the growth of chicks but one possibility is that it acts by means of a true vitamin mechanism. It is conceivable that ascorbic acid cannot be synthesized fast enough by the chick on a highly purified ration, such as has been used in these experiments, to give a maximum rate of growth.

A second possibility is that ascorbic acid stimulates growth indirectly. It may alter the conditions within the intestinal tract to cause more effective utilization of necessary nutrients or to cause greater synthesis of unknown vitamins by intestinal organisms. We know, for example, that feathering is improved by the addition of ascorbic acid to chicks in several of the groups receiving the basal ration with *p*-aminobenzoic acid, indicating that some of the vitamin B_{10} is being synthesized. Further evidence for the indirect action of ascorbic acid is the fact that this compound and *p*-aminobenzoic acid do not give additive effects when fed together on the ration containing solubilized liver.

Another possible mechanism of action is that ascorbic acid in the ration acts as a detoxifying agent. This possibility is unlikely since doubling the levels of solubilized liver or of any of the crystalline vitamins did not depress the growth rate. Also, when the con-

† We are grateful to Miss Marie Zepplin for performing the ascorbic acid assays on the livers.

trol ration was fed with and without ascorbic acid to 2 groups of 3 rats (21 days old) both groups grew equally well for a 5-week period and appeared normal in all respects.

Until we know the mechanism of action of ascorbic acid and other compounds that stimulate growth when fed to chicks receiving such rations as used in these experiments they should be regarded as growth stimulants and not necessarily as specific growth factors. Further studies on these compounds and their mode of action are in progress at our laboratory.

Summary. Ascorbic acid, when fed to chicks receiving various *purified* rations consistently stimulates the growth rate to a small but definite extent. The exact mechanism of action is unknown at present. The relationship of growth effects obtained with ascorbic acid to those obtained with *p*-aminobenzoic acid is compared and discussed.

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A Cross-Protective Reaction Between Moccasin Venom and the Endotoxin of *Salmonella typhimurium*.

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Introduction. We have reported elsewhere[1,2] that mice immunized against the endotoxin of *Salmonella typhimurium* are to a limited extent protected against both lethal and tumor-hemorrhage doses of the endotoxin of *Rhodospirillum rubrum* and *Shigella paradysenteriæ* Flexner, and that a similar cross-protection in respect to the other two organisms is found when the immunization is performed with *Rhodospirillum* or *Shigella*. Further, a characteristic of the endotoxin of gram-negative bacteria generally has been shown to be the ability to induce hemorrhage in transplanted mouse tumors.[3,4] Analogous hemorrhage-induction has been described for venom of the water-moccasin.[5]

The present study was undertaken to determine whether a cross-protection occurs in mice immunized with the endotoxin of a gram-negative bacterium and mice immunized with the ostensibly unrelated hemorrhage-inducing snake venom.

As a representative gram-negative organism, *Salmonella typhimurium* was selected for the source of endotoxin to be compared with the lyophilized venom of the moccasin *Agkistrodon piscivorus*.* The antigenicity of this venom is recognized.[6,7,8]

Experimental. For 10 days several groups of white Rockland male mice were given daily sublethal intraperitoneal injections (about 0.5 LD_{50}) of an aqueous dispersion of the venom. At the end of this period the animals had developed sufficient immunity to survive about 2 LD_{50} amounts of venom. Other groups of mice were immunized with the endotoxin of *Salmonella*. The preparation of this bacterial endotoxin and the dosage schedule employed for the immunization are described elsewhere.[2]

1 Zahl, Paul A., Hutner, S. H., and Cooper, F. S., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 187.

2 Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, in press.

3 Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

4 Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

5 Shimkin, M. B., and Zon, L., *J. Nat. Canc. Inst.*, 1943, **3**, 379.

* Purchased from the Ross Allen Reptile Institute, Ocala, Florida.

6 Githens, T. S., and Wolff, N., *J. Immunol.*, 1939, **37**, 33; 1939, **37**, 47; 1941, **42**, 149.

7 Peck, S. M., and Sobotka, H., *J. Exp. Med.*, 1931, **54**, 407; Peck, S. M., *J. Immunol.*, 1933, **25**, 447; 1934, **27**, 89.

8 Taylor, J., and Mallick, S. M. K., *Indian J. Med. Res.*, 1936, **24**, 272.

TABLE I.

Survival at the end of 48 hours of normal and immunized mice following the interperitoneal injection of various dosages of salmonella endotoxin-containing material and moccasin venom. Numerator = No. of animals surviving. Denominator = total No. of animals used. In each instance the % survival is indicated.

Condition of mice	Amt of salmonella material inj.		Amount of venom injected					
	4 mg	8 mg	.1 mg	.15 mg	.2 mg	.25 mg	.3 mg	.35 mg
Normal	7/22 (31%)	1/24 (4%)	7/12 (58%)	4/18 (22%)	0/15 (0%)	0/13 (0%)	0/14 (0%)	
Salmonella immunized	18/18 (100%)	23/26 (88%)		20/24 (83%)	12/17 (70%)	7/16 (43%)	5/22 (23%)	0/10 (0%)
Venom immunized	16/23 (70%)	9/17 (53%)			14/19 (73%)	18/24 (75%)	6/17 (35%)	0/10 (0%)

The degree of cross-protection was determined by observing survival following injection of varying amounts of venom into mice previously immunized against *Salmonella typhimurium*. Inversely, salmonella endotoxin was similarly injected into the venom-immunized mice.

Results and Discussion. The data listed in Table I indicate that: (1) venom-immunized mice were protected against about 4 LD_{50} of the salmonella endotoxin, and (2) salmonella-immunized mice were protected against about 2 LD_{50} of venom.

Following immunization with the venom, only a limited degree of homologous immunity was developed (Table I). On the other hand, it has been repeatedly demonstrated that injection of the endotoxins of many gram-negative bacteria may confer a resistance to as much as 10-20 LD_{50} of the homologous endotoxin. It may be assumed either that the venom is a poor antigen or that a large proportion of the toxicity of the venom resides in components other than the hemorrhagic. Support of the latter view is furnished by Shimkin and Zon[5] who found it necessary to inject as much as one-half the lethal dose of moccasin venom in order to induce hemorrhage in transplanted tumors. Taylor and Mallick,[8] working with a number of snake venoms, including that of *Agkistrodon piscivorus*, found that while the hemorrhage component of these venoms was intergenerically protective, the neurotoxic components were species-specific. Githens and Wolff[6] also reported a species-specificity of the neurotoxin component and a relative non-specificity for the local necrotic (hemorrhagic) factor in snake venoms, including that of *Agkistrodon*. The proteolytic factor of the venom was not neutralized by antivenin.

The existence of an antigenic relation between moccasin venom and salmonella endotoxin could perhaps have been surmised from the finding of Peck[7] that rabbits injected with the venom of *Agkistrodon piscivorus* became refractory to preparations of gram-negative bacteria which otherwise would have elicited the Sanarelli-Shwartzman response.

A unique property of the free protein por-

tion† of the toxic O antigens of *Salmonella* and *Shigella*, noted by Morgan and Partridge,[9] is an ability to combine with carbohydrate to form highly antigenic complexes whose specificity is determined by the particular carbohydrate used. For instance, blood group A polysaccharide obtained from hog gastric mucin was coupled with shigella protein to form an artificial antigen.[10] This antigen could be used in the production of high-titer A typing sera. The occurrence of a comparable toxic carrier-protein in snake venom could account for the cross-protection observed despite the lack of serological cross-reaction.‡

Summary. Mice immunized with moccasin venom were protected against otherwise lethal doses of the endotoxin of *Salmonella typhimurium;* and, inversely, salmonella-immunized mice were protected against the venom. This cross-protection may be due to the presence in gram-negative organisms and moccasin venom of a common factor characterized by hemorrhagic action, antigenicity, and a lack of serological specificity.

† In an earlier paper of this series[3] we referred to this portion of the toxic O antigen as a polypeptide rather than as a protein. In this usage we followed the early nomenclature of Morgan and Partridge, who have since considered this portion of the antigen to be protein rather than polypeptide.

[9] Morgan, W. T. J., and Partridge, S. M., *Brit. J. Exp. Path.*, 1942, **23**, 151.

[10] Morgan, W. T. J., *Brit. J. Exp. Path.*, 1943, **24**, 41.

‡ The cross-protection as well as the reported hemorrhagic activity of moccasin venom could conceivably have resulted from an inapparent contamination of the venom preparations by a gram-negative bacterium. In an effort to rule out the possibility of contamination, solutions of the venom were tested for agglutination against an O-typing antiserum for *Salmonella typhimurium.* There was no reaction, indicating that the type of cross-protection reported in this paper is not due to carbohydrate-specific antigens held in common by the materials tested. Also, concentrated dispersions of the venom were stained heavily with Loeffler's methylene blue. No evidence of contamination was found.

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Significance of the Protein Level in Synthetic Diets.

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McIntire *et al.*[1] suggest that to achieve optimal growth in rats (males), one or more factors are needed which are deficient in currently used synthetic diets with pure B factors. This deficiency is made up by liver extract. Their basal diet contains 18% purified casein, salts, sugar, fat, fat-soluble vitamins, thiamine, pyridoxine, riboflavin, pantothenic acid, nicotinic acid, and choline. In looking for the missing factor they apparently restrict consideration to the B complex on the assumption that the diet furnishes proper amounts of all other factors which should be fed and because growth is increased when liver extract is given.

We have published observations on dietary protein and growth[2] in which the assumption is made (and substantiated) that the sources of B complex in certain seed meals plus white flour are adequate for optimal growth. A preliminary report[3] dealing with casein levels was based on a similar assumption regarding adequacy of a mixture of rice bran extract, riboflavin, and yeast extract. On the basis of these assumptions both reports point to the

[1] McIntire, J. M., Henderson, L. M., Schweigert, B. S., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 98.

[2] Zucker, T. F., and Zucker, L., *Ind. Eng. Chem.*, 1943, **35**, 868.

[3] Zucker, T. F., and Zucker, L., Abstracts, 105th ACS Meeting (Detroit), April, 1943, Div. Biol. Chem., p. 5B.

fact that the more rapidly growing male requires more protein as expressed in percent protein in the diet than the more slowly growing female; the male level should in the case of casein be put at 24% actual casein, or when weighed out as crude casein, at 27.9% in the diet. A similar conclusion as regards sex difference can be drawn for the chick from the work of Irwin and Kempster.[4]

In the casein level experiments we used crude casein, which is perfectly permissible if our assumption is correct that 5 g rice bran extract, 0.5 g yeast extract, and 100 γ riboflavin per 100 g diet supply all the B factors necessary. Since McIntire's assumption and ours are mutually exclusive, the answer must either be that raising the casein level in the McIntire diet will produce optimal growth, thus making the postulated new factor unnecessary, or the unknown factor is furnished in adequate amounts in crude casein as an impurity when this is raised from 20.9%, which is suboptimal, to 27.9% (N × 6.25 = 18 and 24% respectively). This would demonstrate a fallacy in our conclusion that the male requirement for protein is greater than that of the female.

We have recently observed the growth of females on a diet containing 27.9% Labco casein (N × 6.24 = 24%), otherwise similar to that of McIntire. It was intended for both males and females, but male experiments have not yet been completed. The data (see Fig. 1) indicate that females grow optimally on a diet of 26.7% Labco casein (N × 6.25 = 24%), 67.7% cerelose, 3.6% modified Wesson salts with phosphorus adjusted for casein level, 0.1% carotene in oil, 0.25% wheat germ oil, 1.65% Wesson oil, and the following supplements fed daily: 40 γ thiamine hydrochloride, 50 γ pyridoxine hydrochloride, 80 γ riboflavin, 200 γ calcium pantothenate, 1 mg nicotinic acid, and 10 mg choline hydrochloride. This result fits in very nicely with our assumptions. In terms of McIntire's assumptions, it must be concluded that females do not require the postulated new factor.

It is also seen in Fig. 1 that in male rats on crude casein diets the relation between the growth curve for 20.9% casein and optimal growth (which is achieved on 27.9% casein) is of the same form as that for McIntire's male rats without and with liver. It is also of the same form as the growth of our females on inadequate (N × 6.25 = 15% and below) and adequate protein levels. This demonstrates two other facts. The factor under consideration, if it is not one or more amino acids contained in casein, must be present as an impurity to about the same extent in 7 g crude casein as in 2 g liver extract. Furthermore such a factor has an important nutritional property in common with protein, namely, that the fractional growth deficit decreases as time goes on. McIntire's deficient rats after 3 weeks on experiment have 73% of the weight of those growing optimally with liver supplement. The deficit decreases until after 6 weeks on experiment they show 94% of the weight of the controls. For a discussion of protein and spontaneous realimentation see Zucker *et al.*[5,7]

McIntire *et al.* have shown that the factor is present in yeast in only moderate amounts. It can be only minimally present in rice bran extract, since in our 20.9% crude casein diet on which the growth deficit for males appears, rice bran extract is included in addition to yeast extract. Further, on diets made up of oil seed meals and white flour which support optimal growth in females but result in the same form of male growth deficit already described, the addition of rice bran extract has no effect on the growth, while the addition of fibrin which has been extracted with hot alcohol or of cottonseed flour, restores the male growth to optimal.[2] Therefore the factor is present in cottonseed flour and in fibrin which has been extracted with hot alcohol.

Schneider and Steenbock[8] fed 18% of

[4] Irwin, M. R., and Kempster, H. L., *Mo. Agr. Exp. Sta. Bull.* No. 441, 1942, 3.

[5] Zucker, T. F., Hall, L., Young, M., and Zucker, L., *J. Nutrition*, 1941, **22**, 123.

[6] Zucker, L., and Zucker, T. F., *J. Gen. Physiol.*, 1942, **25**, 445, see esp. graph p. 452.

[7] Zucker, L., Hall, L., Young, M., and Zucker, T. F., *Growth*, 1941, **5**, 399, see esp. p. 409.

[8] Schneider, H. H., and Steenbock, H., *J. Biol. Chem.*, 1939, **128**, 159.

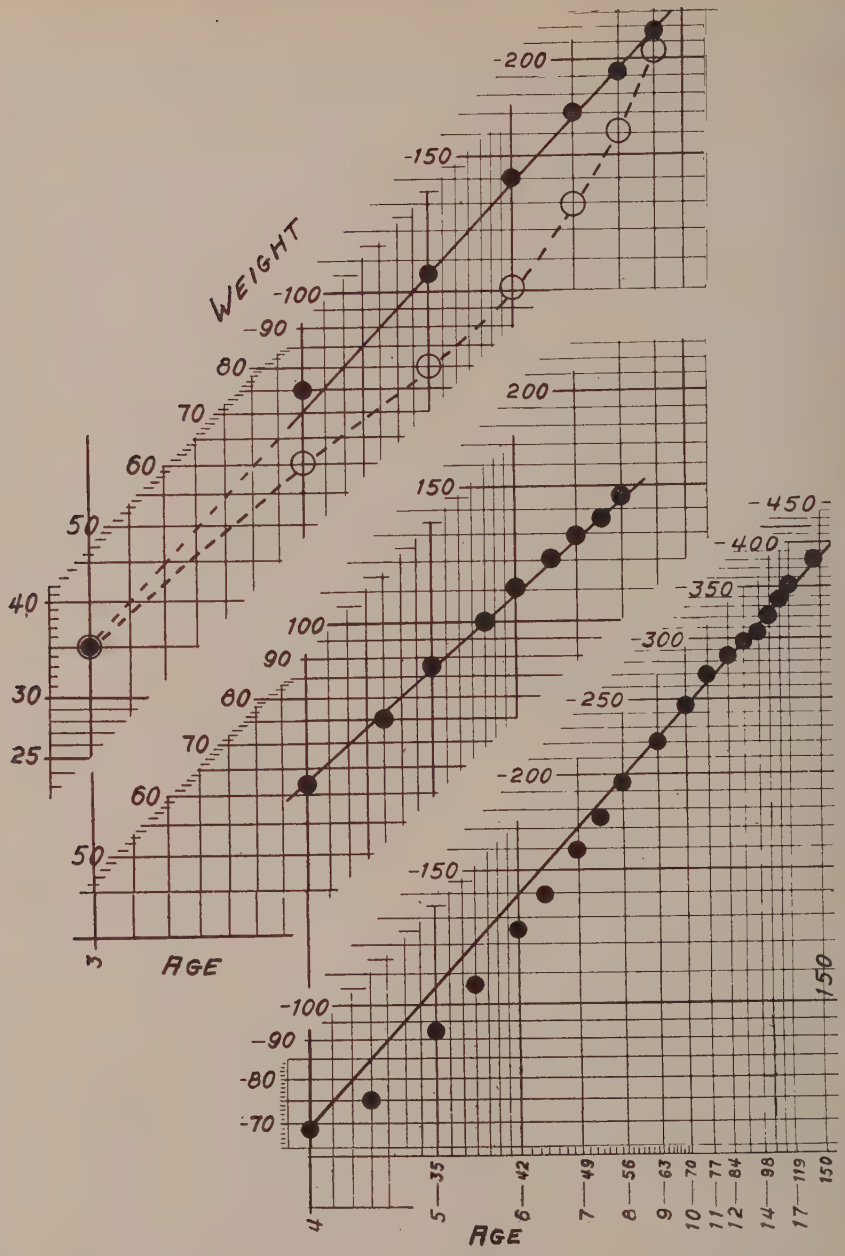

FIG. 1.

Optimal postweaning growth of rats gives a straight line when log weight is plotted against the reciprocal of age.[5,6] The heavy straight lines represent the slopes for male and female growth. The top section shows the McIntire male growth data from 3 weeks on without liver (open circles) and with liver (filled circles). The growth curve with liver agrees with the line for optimal growth. The middle section shows the growth of our females from 4 weeks on, together with the female growth standard. The diet is comparable to McIntire's diet without liver. Females do not appear to need any unknown factor from liver. The bottom

section shows the growth of our males on a suboptimal level of crude casein compared with optimal growth. The typical form of protein (amino acid) deficiency curve, *i.e.*, immediate reduction in growth rate with smooth return towards normal, is common to both top and bottom sections, but the difference in starting age and the difference in the exact protein levels makes the extent of deviation of the experimental growth from the control not strictly comparable.

heated egg albumen. The B factors were derived from rice bran extract and the egg albumen. Their male growth gives an excellent fit to the same line as shown for McIntire's data with 18% casein plus liver extract. Since rice bran extract does not contain the factor in question, egg albumen must either contain more of it than casein, or casein has a less favorable amino acid composition than egg albumen. Both feeding trials and amino acid composition (especially as regards the sulfur amino acids) point to a superiority of egg albumen over casein.

We have thus outlined an experimental basis for judging protein requirement for optimal growth in male and female rats. Experiments now in progress reveal that for 4 weeks there is no significant difference between two groups of male rats on diets with 30% Labco casein (otherwise identical with that of McIntire *et al.*), one with and the other without a supplement of 2% of liver extract. Judging both by our experience and McIntire's results, any existing difference should have reached its maximum by this time. The results will be published in detail when comparable experiments dealing with amino acid supplements to 18% casein have been completed.

Summary. It has been reported by McIntire *et al.* that a diet containing 18% purified casein and 6 pure B factors does not support optimal growth in male rats, and that the missing factor is present in liver extract. Evidence is presented to show that the factor, whatever it is, may not easily be found in the usual concentrated sources of B complex, but rather that it is associated, whether as an impurity or an essential amino acid, with protein of good quality.

14491

Effect of Massive Doses of Thiamine on Fertility and Lactation in the Albino Mouse.*

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The experiments of Perla[1] showed that feeding rats with large amounts of thiamine resulted after one generation in interference with lactation, loss of maternal instinct, cannibalism, and progressive loss of fertility. Continuing this study, Perla and Sandberg[2] found that these symptoms were prevented by giving manganese chloride daily to the animals. If the diet was supplemented with manganese chloride alone, interference with lactation resulted, particularly marked after one generation. The observations of Perla were confirmed by Sure.[3] In contrast to the findings of Perla, and of Sure, Williams and Spies[4] reported normal reproduction in rats kept on stock diets supplemented with large amounts of thiamine through 3 generations. The

* This investigation has been supported in part by a grant from the John and Mary R. Markle Foundation.

1 Perla, D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 169.

2 Perla, D., and Sandberg, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 522.

3 Sure, B., *J. Nutrition*, 1939, **18**, 187.

4 Williams, R. R., and Spies, T. D., *Vitamin B_1 and Its Use in Medicine*, Macmillan Co., New York, 1938, p. 286.

TABLE I.
Data on Reproduction and Lactation in Albino Mice Kept on a Basal Diet of Purina Dog Chow Supplemented with (a) Thiamine, (b) Thiamine + $MnCl_2$, and (c) $MnCl_2$.

Generation	Supplement	No. of mice	No. of litters born	No. of litters weaned
Parent	None	10	10	8
	Thiamine	10	7	7
	" + $MnCl_2$	5	5	4
	$MnCl_2$	5	5	5
F_1	Thiamine, 1st mating	5	5	0
	" 2nd "	5	3	3
	" + $MnCl_2$	5	4	4
	$MnCl_2$	5	4	4
F_2	Thiamine	5	5	4
	" + $MnCl_2$	5	3	3
	$MnCl_2$	5	4	4

present report presents data on the effects of massive doses of thiamine in the mouse. The action of manganese was also studied.

The basal diet consisted of finely ground Purina dog chow. Albino mice of our own strain were divided into 3 groups and fed the following rations: Group 1, basal diet plus 250 mg thiamine per kilo of diet; Group 2, basal diet plus 250 mg thiamine and 1 mg $MnCl_2$ per kilo; Group 3, basal diet plus 1 mg $MnCl_2$ per kilo. The mice were placed on the diets when 21 days old, at which time they weighed 8 to 10 g. The effects of the 3 diets were studied throughout 3 generations.

The growth rate in the 3 groups was practically the same and was similar to that obtained on Purina dog chow alone. In the rat, according to Sure,[3] a supplement of a large amount of thiamine has a stimulating effect on growth. The daily average intake of thiamine for the mice in Groups 1 and 2 during growth was 625-750 μg since the daily food intake amounted to 2.5-3.0 g.

As shown in Table I, reproduction and lactation did not appear abnormal throughout the 3 generations in any of the 3 groups. Second generation mice on the thiamine-supplemented diet destroyed their young upon being mated the first time. However, a subsequent mating saw them successfully wean their young. During reproduction and especially during lactation the intake of thiamine was greatly increased due to the increased food intake. During lactation the mothers consumed as much as 8 g of diet daily. Such an amount of diet would contain 2000 μg of thiamine. The amount of $MnCl_2$ ingested daily during growth was approximately 3 mg, and about 8 mg during lactation.

Summary. The effect of massive doses of thiamine on reproduction and lactation in the albino mouse has been studied. No harmful action was observed.

14492

Influence of Breeding on Induction of Mammary Cancer with Methylcholanthrene in Strain Dba Female Mice.*

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The percutaneous administration of either 9,10-dimethyl-1,2-benzanthracene or methylcholanthrene was reported by Engelbreth-Holm and Lefèvre[1] and Engelbreth-Holm[2] to accelerate the development of mammary cancer in female mice of strain Dba. Morton and Mider[3,4] had previously treated similar mice in like manner, but had not described this phenomenon. The latter authors stated specifically[3] that male and female animals were segregated, whereas Engelbreth-Holm made no definite statement in this regard. This possible difference in technic suggested to the present authors that breeding might condition the response to carcinogens insofar as development of mammary cancer was concerned. Another explanation for the discrepancy in the above findings could be the use of different sub-lines of this strain of mice in the two investigations. It was therefore decided to test the effect of breeding on the carcinogenic induction of mammary cancer within a single sub-line of strain Dba.§ It was first demonstrated that 23 out of 26 untreated breeding females of the sub-line chosen developed mammary cancer spontaneously at an average age of 370 days (range: 300 to 460 days). Only progeny of these breeders were used for the following experiments.

Two groups of female mice 6 to 8 weeks of age were placed on similar diets and given 2 percutaneous applications weekly of a 0.5% solution of methylcholanthrene in benzene. Females of Group I, 27 in number, were allowed to breed, and all animals bore one or more litters during the course of treatment. The 31 animals of Group II were maintained as virgins. Forty-eight male mice were treated similarly with methylcholanthrene.

In Group I all animals developed mammary cancer after an average treatment period of 105 days. The average age of appearance of cancer was thus 155 days, as compared with 370 days in the untreated control breeders. In Group II only 4 out of 31 mice developed mammary cancer. The induction periods for these were 177, 178, 204, and 205 days‖. In contrast, the longest period of induction for any of the carcinogen-treated breeders of Group I was 153 days. Sixty-five percent of the males and virgin females treated developed leukemia; none of the untreated control breeders developed leukemia spontaneously. [Induction of leukemia with carcinogens in strain Dba mice has been reported previously.[1-5]] The average induction period for leukemia was 113-122 days. Probably some of the virgin females of Group II died of leukemia before mammary cancer could have

* This investigation was aided by grants from The Jane Coffin Childs Memorial Fund for Medical Research and the Cancer Fund of the Graduate School of the University of Minnesota.

† Trainee in Radiology, National Cancer Institute.

‡ Division of Cancer Biology of the Department of Physiology.

1 Engelbreth-Holm, J., and Lefevre, H., *Cancer Research*, 1941, **1**, 102.

2 Engelbreth-Holm, J., *Cancer Research*, 1941, **1**, 109.

3 Morton, J. J., and Mider, G. B., *Science*, 1939, **87**, 327.

4 Morton, J. J., and Mider, G. B., *Cancer Research*, 1941, **1**, 95.

§ Sub-line 12 was used in these experiments.

‖ The incidence of mammary cancer in virgin females of sub-line 12 has not been determined. No tumors have appeared as yet in a group of 40 virgin females 9-10 months of age of sub-lines 112 and 212.

5 Law, L. W., *Cancer Research*, 1941, **1**, 564.

TABLE I.
Table Summarizing Data on Induced and Spontaneous Mammary Cancer and Induced Leukemia in Strain Dba Animals of These Experiments.

	Group	No. of mice	No. with mammary cancer	Induction time of mammary cancer, days	No. with leukemia	Induction time of leukemia, days
I.	Breeding ♀	27	27	105	7	106
II.	Virgin ♀	31	4	191	20	113
III.	Untreated breeding ♀	26	23	Mammary cancer appeared spontaneously at an average age of 370 days	0	
IV.	♂	48	0		31	122

appeared. Of the 11 virgins which did not develop leukemia, however, only 4 developed mammary cancer, the induction period of these being longer than in any one of the 27 cases of mammary cancer in treated breeders. It is interesting to note that the incidence of induced leukemia appears to have been reduced in the Group I female breeders, suggesting that either the development of mammary cancer, breeding, or both, might inhibit the induction of leukemia in the same animal. These data are summarized in Table I.

Mammary cancer appeared invariably as multiple growths in the breeding carcinogen-treated animals of Group I. Each of the mammary glands was studded with macroscopic cancerous and precancerous nodules. Only one to 3 mammary cancers appeared in each of the untreated breeding females which developed the disease spontaneously. Macroscopic nodulation did not appear in the noncancerous glands of these mice.

Further experiments are being conducted on the relationship of the estrogenic, genetic, and milk influences to the induction of mammary cancer by chemical carcinogens in various sub-lines of the Dba strain. The nature of the nodulation in all mammary glands of breeding carcinogen-treated females is also being investigated.

Summary. Mammary cancer appeared precociously in strain Dba female mice subjected to percutaneous applications of methylcholanthrene. The latent period was markedly shorter and the total incidence higher in breeding than in virgin animals.

14493

L Type of Growth in Cultures of a Hemolytic Parainfluenza Bacillus.*†

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Colonies similar to the pleuropneumonia group of organisms were isolated and maintained in pure cultures from 2 bacterial species, the *Streptobacillus moniliformis* and *Bacteroides fundiliformis*.[1] The colonies consist of small granules which invade the agar and which swell up on the surface of the colonies to large bodies of 10 micra or more. These large bodies by disintegration again reproduce the small granules. According to the observations of the author, this L type of

* The expenses of this investigation have been defrayed in part by a grant from the Commonwealth Fund.

† This is publication No. 76 of the Robert W. Lovett Memorial Fund for the Study of Crippling Diseases.

[1] Dienes, L., *J. Bact.*, 1942, **44**, 37.

growth originates in both species from the bacteria. Under appropriate conditions the bacteria swell up into large round bodies and germination of granules from these produces the L type of colonies. A similar transformation process of the bacteria was observed in more or less complete form in other species also. The swelling of bacteria into large bodies, the germination of these and the production of tiny colonies consisting of small granules was observed in *B. coli* and *H. influenzæ*. In a flavobacterium and gonococcus the large bacterial forms and the tiny colonies of granules were observed; the latter in the flavobacterium could be grown in 3 successive generations. These processes are so characteristic in appearance and, so far as they can be observed, so similar in different species that they evidently represent a similar transformation of the bacteria with the difference that in most cases we are not able to provide the conditions necessary for the continuous growth of the granular form.

Large swollen forms are often present, in addition to the species already mentioned, in cultures belonging to various species cultivated directly from the human body. Meningococci isolated from spinal fluid often swell up to such forms and they are not rare in streptococcus cultures. The development of L type of colonies was not observed in these species. From the common saprophytic organisms of the human body parainfluenza bacilli produce large bodies most often and recently the development of L type of growth from these large bodies was observed.

The strains belonging to the parainfluenza bacilli are very variable. The colonies consist sometimes of regular-shaped individual bacilli, sometimes of long segmented filaments which may be regular or may develop into large fusiform or round bodies. The pleomorphic colonies of this bacillus can be seen in the majority of throat cultures if they are appropriately examined, and a colony transformed entirely into large round bodies was illustrated in a former paper.[2] The pleomorphism usually disappears after a few transplants.

During the last 5 years many pleomorphic colonies were transplanted to horse blood and ascitic agar plates, but the large bodies always disappeared without further development. Since last August the author carried in his throat a very pleomorphic hemolytic parainfluenza strain. The large bodies of this strain as in other strains did not show any development on horse blood and ascitic agar plates. In December, experimenting with new media, the pleomorphic colonies were transplanted to a medium consisting of sedimented boiled blood agar, horse blood, and fresh egg yolk. On this medium after 4-8 hours' incubation many of the transplanted large bodies considerably increased in size and they were deeply stained in agar preparations with methylene blue and azur. From many of these large bodies small light blue staining granules grew into the media. These germinating large bodies were similar in every respect to the corresponding structures formerly described in *Streptobacillus moniliformis* and *B. coli* cultures. The largest size to which the L type of growth developed corresponds about to Fig. 8, Plate I of the aforementioned paper.[1] After 6-8 hours the growth did not develop and the granules started to lose their staining and disappeared. After 24 hours' incubation the plates were usually overgrown by bacterial colonies.

The difference between the plates on which the L type of growth did or did not develop was marked. When L growth did not develop the large bodies remained stationary; after 6 hours most of them were vacuolized and they were stained only at the periphery or not at all. On the plates on which L type of growth developed the deeply and uniformly stained large bodies were conspicuous. The medium on which the development of L was observed, although better than those tried before, is insufficient for the continuous growth of the L. A similar germination of the large bodies with consecutive disappearance of the tiny colonies was observed in cultures of *Streptobacillus moniliformis* on certain media not appropriate for the growth of L_1.

Several circumstances under which this observation has been made are of interest. L type of growth appeared on a new medium

2 Dienes, L., *J. Inf. Diseases*, 1939, **69**, 24.

after many trials during a period of five years had been unsuccessful. The development of large forms under normal conditions of growth probably indicates always the tendency for the transformation into L type, but in many cases our culture methods are inappropriate to induce germination of the large bodies as well as to allow continuous growth of the L forms. All strains in which L type of growth was formerly observed were cultivated from pathological processes. The case described in this note is the first in which this growth developed in a culture obtained from a normal human throat. Influenza bacilli cultivated from the throat have never shown L type of growth. The parainfluenza strains are similar in many respects to the *Streptobacillus moniliformis* which is also a normal inhabitant of the rat's throat.

The L type of colonies developing in the culture of parainfluenza, influenza bacillus, and of gonococcus are inconspicuous and transitory. However, their development indicates that those processes, the full development of which was observed in *Streptobacillus moniliformis* and in *Bacteroides fundiliformis*, are present also in these species. The significance of these processes in the life of the bacteria and in their pathological effects are not known, but such deep-going transformation of the bacteria cannot be without significance.

Summary. In a hemolytic parainfluenza strain a transformation process of the bacteria similar to that occurring in *Streptobacillus moniliformis* and *Bacteroides* were observed. The bacteria swelled up into large round bodies and these, transferred to appropriate media, germinated and produced a tiny colony consisting of granules which invaded the agar. The tiny colonies of granules were transitory and after 8 hours' incubation ceased to grow and disappeared.

14494

A Method of Obtaining Blood Samples Directly from the Hepatic Vein in Man.

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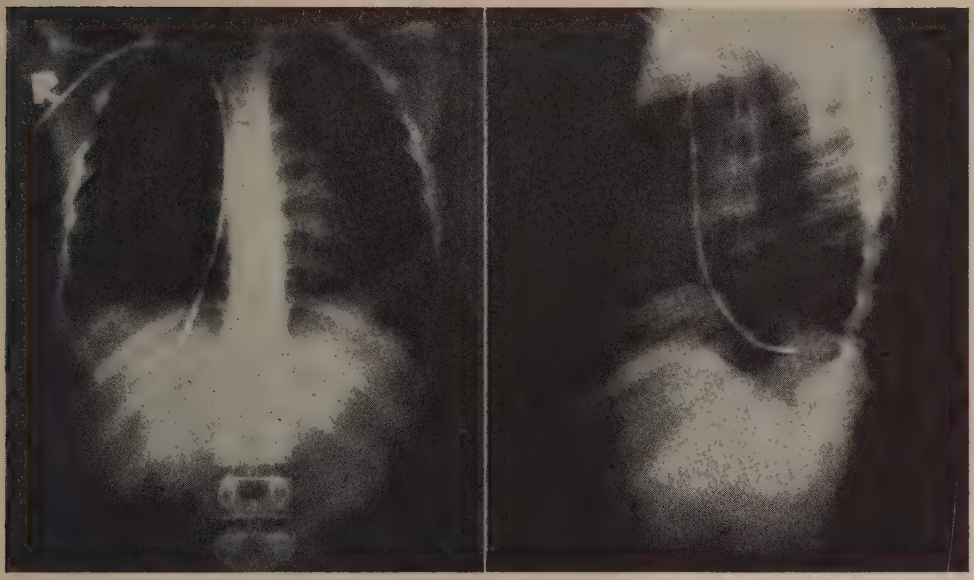
Studies regarding the function of the liver in man have been hampered by the inability to obtain blood either immediately before or after its passage through the liver. In the course of observations on the circulation, during which blood samples were being obtained from the right side of the heart through a catheter in the venous system, we have observed that the tip of the catheter can often be manipulated into one of the hepatic veins, enabling us to withdraw blood from these vessels. The purpose of this note is to call attention to this as a means of obtaining blood directly from the hepatic vein in man.

The liver receives blood from the portal vein and the hepatic artery. The blood from both of these vessels drains into the hepatic sinusoids, which in turn unite to form the central veins. These come together to make up the hepatic veins, usually 2 or 3 in number, which pass upward to enter the inferior vena cava only a short distance below its entrance into the heart. On rare occasions the hepatic veins may empty directly into the right auricle. No other major veins occupy a position in this locality. Roentgenologists[1] have noted that the hepatic veins may at times be seen crossing the angle formed by the right border of the heart and the diaphragm.

The passage of a catheter through the

[1] Roesler, Hugo, *Clinical Roentgenology of the Cardiovascular System*, C. C. Thomas Co., Baltimore, Md., 1937.



A B

FIG. 1.

X-ray film studies showing catheter in the hepatic vein. A. Antero-posterior view. B. Right lateral view.

venous system to the right auricle was first carried out in man by Forssmann.[2] Since then various uses of the catheter technic have been reported, but it has remained for Cournand and his associates[3,4] to demonstrate the utility and safety of the procedure. They have now studied several hundred patients without serious accident. We have carried out the procedure in 75 instances without mishap.

The method for obtaining blood from the hepatic vein is simply an extension of that developed by Cournand *et al.*[3,4] for obtaining blood from the right auricle. We have carried out the procedure on an X-ray fluoroscopic table. The catheters used are No. 8 or No. 9 radiopaque ureteral catheters approximately 80 cm long.* A slight permanent angulation about 4 cm from the tip to about 30° from linear has proven to be very useful. At all times while the catheter is in place a slow drip of physiological saline solution is passed through it. One must constantly be vigilant that the flow of saline is continued, because clotting within the catheter will occur if the flow is stopped. Before blood samples are withdrawn for analysis a small amount must be withdrawn and discarded so that the sample will not be contaminated by the saline in the catheter.

With local procain anesthesia a small incision is made over the basilic vein in either antecubital space. The cephalic vein is not used because of frequent difficulty in passing the catheter over the course of this vein. The vessel is isolated and a transverse slit made in its wall. The tip of the catheter is introduced into the vein and passed up the arm and into the auricle under fluoroscopic guidance. The catheter is then rotated so that the curvature of the tip is directed to the patient's right. It is then advanced downward and should pass out of the auricle into the inferior vena cava. If the catheter enters

[2] Forssmann, W., *Klin. Wschschr.*, 1929, **8**, 2085.

[3] Cournand, Andre, and Ranges, Hilmert A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 462.

[4] Cournand, A., Riley, R. L., Bradley, S. E., Breed, E. S., Noble, R. P., Lauson, H. D., Gregersen, M. I., and Richards, D. W., *Surgery*, 1943, **13**, 964.

* Obtained from the United States Catheter and Instrument Co., Glens Falls, New York.

the hepatic vein it will almost immediately be seen to pass to the right and downward into the right upper quadrant of the abdomen. The position at this time is quite distinctive, as shown in Fig. 1. Instead of passing into the hepatic vein the catheter may pass into the right ventricle or almost straight downward in the inferior vena cava. In either event it may be returned to a higher level, rotated, and further attempts made to manipulate the tip into the hepatic vein. At all times the manipulations should be gentle, force never being used.

As pointed out by Cournand and Ranges,[3] the catheter may not always pass in the desired direction, but on repeated manipulation we have been able to reach the right auricle in over 90% of the cases attempted. The most frequent cause of failure has been spasm of the peripheral vein, which makes rotation and movement of the catheter difficult and painful. A rest period and additional local anesthesia at the site of insertion may relieve this. If the auricle has been reached, and there is little or no spasm, we have been able to enter the hepatic vein in most instances, but failure may occur if the catheter repeatedly passes into the right ventricle or the inferior vena cava and cannot be guided into the hepatic vein.

On removal of the catheter the skin is closed with several silk sutures, but the vein is not tied off. If bleeding occurs, it is controlled with a pressure bandage. A small linear venous thrombosis may develop locally, but in most instances the vein recanalizes within 2 or 3 weeks.

Although this was not the primary purpose of our work, we have made a few observations on hepatic vein blood. The hematocrit reading and hemoglobin content of blood from the liver did not differ from simultaneously obtained arterial blood specimens. The oxygen content of the hepatic vein blood in 6 patients has been somewhat lower than that of mixed venous blood obtained from the right auricle. In the fasting subject the non-protein nitrogen content was not significantly different from arterial blood, while the glucose level was only slightly lower in hepatic vein blood.

In addition to the above data we have made observations upon the bacterial colony counts in hepatic vein blood in patients with subacute bacterial endocarditis. This work, done in collaboration with Dr. Paul B. Beeson, is to be reported in detail elsewhere.

Summary. A method is described for obtaining samples of blood from the hepatic vein in man by means of a catheter in the venous system. Only a moderate amount of skill is required, and if carried out with care, samples can be obtained with minimal discomfort and danger to the subject.

The authors are deeply indebted to Dr. Andre Cournand and his associates for the fundamental training in the methods utilized in this work.

14495

Delayed Contraction of Denervated Muscle with Intravenous Barbiturates.*

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It is a known fact that denervated striated muscle contracts when in contact with cholinergic substances that have nicotinic properties. Intravenous injections of minute doses of acetylcholine and certain carbominocholine derivatives produce a contraction of denervated facial muscles within the circulation time.[1] Large doses of epinephrine also cause the denervated facial muscle to contract, but

* This work has been partly aided by a grant from the Josiah Macy, Jr., Foundation.

1 Bender, M. B., Spirtes, M. A., and Sprinson, D. B., *J. Pharm.*, 1943, **77**, 107.

this does not occur until 60 to 90 seconds after the intravenous injection. Such delayed muscular contraction is probably the result of a secondary, rather than direct effect of epinephrine.[2]

In the course of these experiments, it was noted that intravenous sodium pentobarbital anesthesia produced a delayed contraction of the denervated facial muscle in 11 of 12 tested monkeys. The contraction appeared after a latency varying from 16 to 60 seconds following the administration of 25 mg of sodium pentobarbital per kilo of body weight. The contraction could be elicited only by the intravenous route with a speed of not less than 10 mg per second. So long as the injection was rapid, the effect could be obtained even with subanesthetic doses.

The contraction induced with sodium pentobarbital lasted from 40 to 75 seconds. It could be so elicited so long as the muscle remained denervated. It was slightly enhanced by eserine and never blocked by atropine.

Similar results, and under the same conditions, were obtained with other barbiturates, such as sodium thiopentobarbital, sodium amytal, and sodium evipal. Intravenous injection of other anesthetics, such as paraldehyde, urethane, or ether inhalation did not produce contraction in the denervated face.

The mechanism of this contraction is difficult to explain. Barbiturates have no significant direct stimulation effect on striated muscle *in vitro*.[3] This, and the fact the contraction is delayed, suggest that the effect is indirect and secondary. It is possible that the rapid intravenous administration of barbiturate causes a physiologic disturbance in the body leading to the formation or release of substance causing the denervated muscle to contract. The nature and site of formation of this hypothetical substance are obscure. Because of its contractile effect on denervated muscle, the substance may be related to the cholinergic group.

[2] Bender, M. B., *Am. J. Physiol.*, 1939, **126**, 430.

[3] Goodman, L., and Gilman, A., *The Pharmacological Basis of Therapeutics*, Macmillan Co., N.Y., 1941.

14496 P

Genetic and Certain Non-genetic Factors with Reference to Leukemia in the F Strain of Mice.*

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MacDowell and Richter[1] found that the incidence of leukemia in hybrids could be roughly correlated with the total heredity from the high leukemia C58 strain when C58 mice were crossed with low leukemia StoLi animals. A "maternal influence" was suggested, for if the female parent was of the C58 strain the incidence of leukemia was greater than in the reciprocal cross. Cole and Furth,[2] working with the Ak and Rf stocks, concluded that susceptibility to leukemia is probably inherited as a multiple factor character, and is influenced by undetermined environmental factors. In various crosses the common logarithm of the percent leukemia was a simple function of the percent heredity from the high leukemia stock. Female mice had a higher incidence of leukemia than males. It was

* This investigation was aided by grants from The Jane Coffin Childs Memorial Fund for Medical Research and the Cancer Fund of the Graduate School of the University of Minnesota.

[1] MacDowell, E. C., and Richter, M. N., *Arch. Path.*, 1935, **20**, 709.

[2] Cole, R. K., and Furth, J., *Cancer Research*, 1941, **1**, 957.

TABLE I.

Table Summarizing the Data on Matings Between the High Leukemia F Strain and Strains A, CBA, and C57 (Low Leukemia Strains). Results were essentially the same when strain F mice were crossed with any one of the 3 low leukemia strains. The figure listed for each 100-day period gives the number of mice per 100 animals developing leukemia in that portion of the life span.

Age, days	High leukemia (F strain)	Backcross to high leukemia strain*	F_1 hybrids†	Backcross to low leukemia strain‡	F_2 hybrids
0- 100					
100- 200	3	2			
200- 300	7	9	0.5	1	1
300- 400	14	10	0.5	1	5
400- 500	13	10	6	1	7
500- 600	13	10	7	3	6
600- 700	3	9	11	11	6
700- 800		6	9	11	3
800- 900		0.5	8	3§	3
900-1000			7	1	6
1000-1100			2		
No. of mice	325	155	216	117	68

* Nine mice beyond 700 days of age still living.

† Fifteen animals living—over 900 days of age.

‡ Sixteen mice over 700 days of age still living.

§ Thirteen non-leukemic animals have died of polycystic kidney disease which is inherited as a recessive character from strain A.

concluded that the genetic basis for spontaneous leukemia may vary for different stocks of mice, since Mercier[3] had observed that susceptibility to spontaneous lymphosarcoma was inherited as a simple Mendelian recessive character, and the results of MacDowell and Richter were somewhat at variance with those of Cole and Furth. In later experiments with the Ak stock Furth, Boon, and Kaliss[4] found that when animals of this stock were crossed with the C3H rather than the Rf stock the incidence of leukemia in F_1 hybrids was higher, approximating that in the Ak stock, if the female parent was of the latter stock (50 as compared with 58%); the incidence of leukemia was 34% in F_1 hybrids of the reciprocal cross.

The present study was carried out using the F strain of mice as the high leukemia stock.[5] Of 325 control animals there were 173 cases of leukemia, or an incidence of 53%. Mice of the F strain were crossed with animals of strains A, CBA, and C57, in which the incidence of leukemia is less than one percent. Data on the breeding experiments are summarized in Table I.

In F_1 hybrids and backcrosses to the high leukemia stock the incidence of leukemia was approximately the same as in the pure F stock; in the backcross to the low leukemia stock the incidence of leukemia was decreased (Table I). The most significant finding would seem to be the delayed appearance of leukemia in the hybrid animals; this retardation was observed by MacDowell and Richter, and by Furth and his co-workers, although given little emphasis. Whether the delayed appearance of leukemia is to be correlated with the degree of inheritance from strain F or with the increased life expectancy in hybrids is difficult to say. The greater the number of strain F genes, the earlier leukemia appeared. However, life expectancy in F_1 hybrids was greater than in backcrosses to the F strain; leukemia also appeared later in life, but in approximately the same percent. For the heterogeneous F_2 population the appearance of leukemia was scattered through the various age groups. One of the observations which might indicate that the correlation is not entirely with life expectancy is that in backcrosses towards the low leukemia stock the life expectancy is not as great as in F_1 hybrids—still the first cases of leukemia appeared later than in F_1 hybrids. It might be that hybrids possess a greater degree of resistance towards

[3] Mercier, L., *C. R. Soc. de Biol.*, 1940, **133**, 29.

[4] Furth, J., Boon, M. C., and Kaliss, N., *Cancer Research*, 1944, **4**, 1.

[5] Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1939, **37**, 400.

the unknown causative "agent" of leukemia, and consequently the disease appears later in life and in a more chronic form. With an increased life expectancy metabolic differences might be expected to delay the onset of neoplastic change.

Concerning the incidence of leukemia in males as compared with females, in both hybrids and the pure F stock there was no significant difference in either the total incidence or the age distribution. Although administration of estrogens results in an increased incidence of leukemia in low leukemia stocks,[6] there is no evidence that sex hormones play a role in the spontaneous appearance of leukemia in strain F mice. Fifty-six strain F mice were gonadectomized at weaning age (34 males and 22 females). When no mouse was older than 525 days of age 17 of the castrate males and 10 of the 22 spayed females had developed leukemia. Gonadectomy did not alter the incidence of leukemia in either sex; the castrate atrophy of the male and female accessory sex organs indicated that the adrenal glands were not an extragonadal source of appreciable amounts of sex hormone.

There was no evidence for the existence of a leukemia "milk influence." Previous experiments on foster-nursing in this strain[7] indicated that a maternal influence, as for mammary cancer, is not operating.

[6] Gardner, W. U., Kirschbaum, A., and Strong, L. C., *Arch. Path.*, 1940, **39**, 1.

[7] Kirschbaum, A., and Strong, L. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 404.

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Comparative Fibromatogenic Action of Ovarian and Urinary Estrogens.*

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So far all estrogens, natural or artificial, free or esterified, have been shown to induce abdominal fibroids when continuously administered to female guinea pigs for a certain time.[1,2] The urinary estrogen, estriol, also has been found to be fibromatogenic[3] though less so than estradiol or estrone, whereas with equilenin fibromatogenic action was insignificant[4] even when quantities twice or thrice those of estrone were absorbed. On the other hand, great increase of uterine weight and formation of endometrial polyps was also obtained with equilenin under these experimental conditions.[4] Equilenin has also been found to be less active than estrone in eliciting mammary adenocarcinoma in mice.[5]

One might tentatively suggest that presence of hysterotoxic and epitheliotoxic actions in the absence of fibromatogenic ones in the

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[1] Iglesias, R., Tesis Universidad de Chile, 1938, *Public. Med. Exp.* (Chile), No. 1; Lipschütz, A., and Iglesias, R., *C. R. Soc. Biol* (Paris), 1938, **129**, 519; Lipschütz, A., and Vargas, L., *Lancet*, 1939, **1**, 1313.

[2] Lipschütz, A., *J. Am. Med. Assn.*, 1942, **120**, 171; *Cold Spring Harbor Sympos.*, 1942, **10**.

[3] Szabó, E., *Rev. Chil. Hig. y Med. Prev.*, 1940, **3**, 127.

[4] Thibaut, R., Tesis Universidad de Chile, 1941, *Public. Med. Exp.* (Chile), No. 7; Lipschütz, A., Thibaut, R., and Vargas, L., *Cancer Research*, 1942, **2**, 45.

[5] Lacassagne, A., *Ergebn. d. Vitamin-u. Hormonforsch.*, 1939, **2**, 259.

TABLE I.

Ninety-one Castrated Female Guinea Pigs with Subcutaneously Implanted Small Pellets of Ovarian Estrogens and Big Tablets of Urinary Ones. Necropsy at 90 days after implantation.

Estrogens implanted	Absorbed per day, avg. μg	Fibrous tumoral effect F.T.E. avg*	No. of animals	No. animals reaching avg F.T.E. of estradiol group	No. tumoral marks of classes 2 and 3 per animal
Alpha-estradiol	19	4.9	10	4	1.4
Estrone	12.5	3.2	27	8	0.9
Estriol	111	4.3	15	6	1.2
Equilenin	97	3.7	15	6	1.3
Alpha-dihydroequilenin	111	4.8	12	5	1.5
Beta-dihydroequilenin	136	1.7	12	2	0.5

* For the system of classification and units see Lipschütz and others.[6]

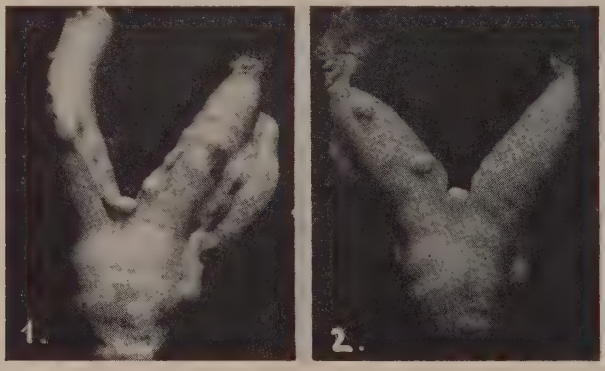

FIG. 1.

Uterus of guinea pig with subcutaneously implanted tablet of alpha-dihydroequilenin. Absorption of 136 μg per day in the course of 90 days. Subserous and parametrial tumors. Nat. size.

FIG. 2.

Absorption of 131 μg of beta-dihydroequilenin per day in the course of 90 days. Subserous and parametrial tumors. Nat. size.

guinea pig treated with equilenin could be due to the different thresholds upon which these different actions depend. If so, fibromatogenic action should be attained with equilenin as well as other less active estrogens, if sufficient quantities are continuously administered.

Comparative experiments were made with 6 different estrogens. Small pellets of estradiol or estrone of 1.6 mm in diameter weighing about 3.5 mg were used for subcutaneous implantation, as well as tablets of estriol, equilenin, alpha-dihydroequilenin and beta-dihydroequilenin§ of 5 mm in diameter weighing 20 to 43 mg. Under these experimental conditions absorption in the estriol-equilenin and dihydroequilenin groups was 5 to 10 times greater than in the estradiol-estrone group. All animals were sacrificed 90 days after subcutaneous implantation. Results are given in Table I.

The fibrous tumoral reaction with an average of 19 μg of estradiol was of the usual degree and the average reaction was reached in 4 out of 10 animals. A smaller reaction was

§ With a single subcutaneous injection of 50 μg of alpha- or beta-dihydroequilenin in oil full estrus was produced only in 2 out of 10 castrated rats; most of animals reached only proestrus. Unpublished work of R. F. Mello and C. Franke in this Department.

[6] Lipschütz, A., Bellolio, P., Chaume, J., and Vargas, L., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164.

obtained with only 13 μg of estrone. With 97 to 111 μg of estriol, equilenin and alpha-dihydroequilenin the result was in every respect equal to that with 19 μg of estradiol: there was the same average fibro-tumorous effect (F.T.E.), the same percentage of animals reaching the average F.T.E. of the estradiol group, and the same number of tumoral marks of classes 2 and 3 per animal. A slight reaction was obtained with beta-dihydroequilenin, but here again there were 2 animals with a F.T.E. = 5.5.

In our previous experiments no fibromatogenic action was seen when small quantities of equilenin were used; the present experiments show that the use of large quantities of equilenin will cause fibromas. A tentative conclusion is offered that the body tends to protect itself against the tumorigenic action of estrogens by the conversion of the more active substances (*e.g.*, estradiol, estrone) into less active ones (*e.g.*, estriol, equilenin). The differences in renal threshold for these substances may also be important.

Summary. Abdominal fibroids were induced in female guinea pigs with the natural urinary estrogen, equilenin, and two hydrogenated ones as alpha- and beta-dihydroequilenin. Estrogenic activity, even though it may be weak, is always concomitant with the fibromatogenic activity. Beta-dihydroequilenin has been shown to be the least fibromatogenic estrogen.

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